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FOREWORD

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Margaret J Wheelbar 6/18/00
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Introduction

This is the second year report for my three year IDEA Award. The purpose of this award was to initiate a breast cancer project in my lab. My lab has been focused for many years on the role of cadherin cell adhesion molecules in cellular behavior. Cadherins are cell-surface proteins that play important roles in normal cell-cell interactions. Members of the cadherin family of transmembrane glycoproteins mediate calcium-dependent, homotypic cell-cell adhesion. Numerous studies have implicated E-cadherin as a tumor suppressor protein in carcinomas; i.e., decreased E-cadherin adhesion correlates with a tumor phenotype. The mechanism by which the adhesive function is decreased varies amongst different tumors, but disruption of the function of E-cadherin, regardless of the mechanism, is thought to aid in the formation of a tumor. With this award, I have been able to focus some of my efforts on studying similar questions with regard to human breast cancer cells. Two graduate students focused their efforts on getting the breast cancer project off the ground. Marvin Nieman has been a Ph.D. student in my lab for 4 years and has been studying the effect of dominant-negative cadherins on squamous epithelial cells. He finished up that project and moved on to the breast cancer project. Ryan Prudoff is a masters student in the lab who spent a year working with Marvin on a survey of a large number of breast cancer cell lines for expression of cadherins. In addition, technical help has been provided by Christine Trapp. The effort of these individuals has contributed to our understanding of how cadherin-mediated cell interactions may influence the behavior of breast cancer cells. In particular, our lab has shown that N-cadherin, which is not normally expressed by epithelial cells, promotes motility and invasion when expressed by human breast cancer cells. Both Marvin Nieman and Ryan Prudoff graduated and their work will be continued by Young Kim and Emhonta Johnson.

Body

At the end of this section, I have copied the portion of the Statement of Work from the original proposal that is appropriate for the first 2 years of this project and have indicate in green those tasks which were completed last year and in red those tasks which have been completed this year.

1. Introduction to the study

Cadherins constitute a family of transmembrane glycoproteins that mediate calcium-dependent homotypic cell-cell adhesion and play an important role in the maintenance of normal tissue architecture. The cadherin intracellular domain interacts with several proteins collectively called catenins that link cadherins to the actin cytoskeleton (reviewed in Wheelock et al., 1996). This linkage is required for full cadherin adhesive activity. Either β -catenin or plakoglobin binds directly to the cadherin and to α -catenin, while α -catenin links directly and indirectly to actin (Aberle et al., 1994; Nagafuchi et al., 1994; Stappert and Kemler, 1994; Knudsen et al., 1995; Rimm et al., 1995; Nieset et al., 1997; Watabe-Uchida et al., 1998). Their ability to simultaneously self-associate and link to the actin cytoskeleton enables cadherins to mediate both the cell recognition required for cell sorting and the strong cell-cell adhesion needed to form tissues.

In addition to their structural role in the adherens junction, catenins are thought to regulate the adhesive activity of cadherins. For example, phosphorylation of β -catenin in Src transformed cells may contribute to the non-adhesive phenotype of these cells (Matsuyoshi et al., 1992; Hamaguchi et al., 1993). In addition, p120^{cas}, originally identified as a Src substrate and subsequently shown to bind to the cytoplasmic domain of cadherins, has been suggested to play a role in regulating the adhesive activity of cadherins (Reynolds et al., 1994; Shibamoto et al., 1995; Daniel and Reynolds 1995; Aono et al., 1999; Ohkubo and Ozawa, 1999).

Numerous studies have demonstrated the importance of the E-cadherin/catenin complex in maintaining the normal phenotype of epithelial cells. Early studies showed that inhibiting E-cadherin activity with function-perturbing antibodies altered the morphology of MDCK cells and conferred upon them the ability to invade both collagen gels and embryonic chicken heart tissue (Behrens et al., 1989; Chen and Öbrink 1991). In addition, invasive, fibroblast-like carcinoma

cells could be converted to a non-invasive phenotype by transfection with a cDNA encoding E-cadherin (Frixen et al., 1991). Moreover, E-cadherin expression is down regulated or lost in epithelial tumors from various tissues, including stomach, colon, head and neck, bladder, prostate and breast (Mayer et al., 1993; Dorudi et al., 1993; Schipper et al., 1991; Bringuier et al., 1993; Umbras et al., 1994; Oka et al., 1993).

It has been suggested that alterations in cadherin function may be a critical step in the development of breast cancers. A survey of 18 cell lines derived from breast carcinomas showed that ten lines failed to express detectable levels of E-cadherin and two other lines failed to express α -catenin (Pierceall et al., 1995). Other studies have identified breast tumor cell lines with mutations in the E-cadherin gene (Berx et al., 1995) or with changes in the levels of expression or in the phosphorylation state of β -catenin or plakoglobin (Sommers et al., 1994). Surveys of breast cancer tissue make an equally compelling case for the involvement of E-cadherin in the formation or progression of breast tumors and clinical studies have shown that loss of E-cadherin correlates with metastatic disease and poor prognosis (Oka et al., 1993; Guriec et al., 1996; Moll et al., 1993; Gamello et al., 1994; Rasbridge et al., 1993; Berx et al., 1996).

In vitro studies support the role of E-cadherin as an invasion suppressor gene. For example, forced expression of E-cadherin in rat astrocytoma cells suppressed motility (Chen et al., 1997). Likewise, transfection of invasive E-cadherin-negative breast or prostate cell lines with mouse E-cadherin resulted in cells that were less invasive in *in vitro* assays (Frixen et al., 1991; Luo et al., 1999). When treated with function blocking E-cadherin antibodies, the transfected cells returned to an invasive phenotype thus implicating E-cadherin as an invasion suppressor (Frixen et al., 1991).

Although a number of studies with breast carcinoma cell lines have shown that loss of E-cadherin generally results in an invasive phenotype, important exceptions have been reported. In one study, two E-cadherin-negative cell lines were shown to be noninvasive (Sommers et al., 1991). These authors suggested that in order for E-cadherin-negative cells to be invasive they must also express vimentin.

In another study, Sommers et al. (1994) showed that transfection of E-cadherin into the invasive breast cancer cell lines, BT549 and HS578t altered neither the morphology nor the invasive behavior of these cells. These authors speculated that the transfected E-cadherin may not be fully functional in these cells due to altered post-translational modification of the cadherin-associated proteins β -catenin, α -catenin or plakoglobin.

It has been suggested that, unlike E-cadherin, N-cadherin may promote motility and invasion in carcinoma cells. For example, Hazan et al. (1997) reported that expression of N-cadherin by breast carcinoma cells correlated with invasion and suggested that invasion was potentiated by N-cadherin-mediated interactions between the breast cancer cells and stromal cells. A study conducted in our laboratory suggested that N-cadherin may play a more direct role in the process of invasion and may actually promote invasion by inducing a scattered phenotype when expressed by oral squamous cell carcinoma-derived cells (Islam et al., 1996). In this study, forced expression of N-cadherin resulted in down-regulation of endogenous E- and P-cadherins making it impossible to separate the motility-promoting effects of N-cadherin from the motility-suppressing activity of E-cadherin. In contrast, it has been suggested that N-cadherin promotes contact inhibition in normal skeletal muscle myoblasts and in so doing inhibits migration upon contact but does not suppress motility in subconfluent cells (Huttenlocher et al., 1998).

Thus, the information in the literature concerning the role cadherins may play in tumor cell invasion is inconclusive and even contradictory, prompting us to revisit the question using new reagents generated by our laboratory to examine both previously studied and newly derived breast cancer cell lines. The data presented in this paper indicate: 1) Decreased expression of E-cadherin does not necessarily correlate with invasion in breast cancer cells; 2) N-cadherin expression correlates both with invasion and motility in breast cancer cells and likely plays a direct role in promoting

motility; 3) Forced expression of E-cadherin in invasive, N-cadherin positive cells does not reduce their motility or invasive capacity; 4) Forced expression of N-cadherin in non-invasive, E-cadherin-positive cells produces an invasive cell even though these cells continue to express high levels of E-cadherin; 5) The data suggest that N-cadherin mediated cell motility may be stimulated by fibroblast growth factor receptor signaling.

2. Materials and Methods

Cells: Breast carcinoma cell lines were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (SKBr3, MDA-MB-435, MDA-MB-436, BT-549, and Hs578t) or minimal essential medium (MEM) with 10% fetal bovine serum (MDA-MB-453 and BT-20). The cell lines MCF-7 and MDA-MB-231 were obtained from Dr. Mary J. C. Hendrix (University of Iowa, Iowa City, IA) and maintained in DMEM with 10% fetal bovine serum. The cell lines SUM159PT and SUM149 were obtained from the University of Michigan Human Breast Cell/Tissue Bank and Data Base and maintained in Ham's F-12 with 5% fetal bovine serum supplemented with insulin (5 mg/ml) and hydrocortisone (1 mg/ml). The cell line SUM1315 was obtained from the University of Michigan Human Breast Cell/Tissue Bank and Data Base and maintained in Ham's F-12 with 5% fetal bovine serum supplemented with insulin (5 mg/ml) and epidermal growth factor (10 ng/ml). HT1080 cells were obtained from ATCC and maintained in DMEM 10% fetal bovine serum.

Transfections: To transfect MDA-MB-435 with E-cadherin, the calcium phosphate transfection kit (Stratagene, LaJolla, CA) was used according to manufacturer's protocol. For electroporations (BT-20 cells), 1×10^6 cells were washed with PBS and resuspended in electroporation buffer (120 mM KCl, 0.15 mM CaCl_2 , 10 mM K_2HPO_4 , 10 mM KH_2PO_4 , 25 mM HEPES, 2 mM EGTA, 5mM MgCl_2) supplemented with 2 mM ATP and 5 mM glutathione. Following a 5 min incubation on ice, the cells were electroporated at 500 μF and 380 V in a BioRad Gene Pulser (BioRad, Richmond, CA). Cells were immediately plated in a 100 mm dish in complete medium. Floating cells were removed and fresh medium was added 24 h after electroporation; puromycin was added to the culture for selection of clones 48 h after electroporation.

Clones and vectors: For expression of N-cadherin in MDA-MB-435 and BT-20 cells, a restriction fragment containing nucleotides 442 to 3362 (GenBank accession S42303, a kind gift of Dr. Avri Ben-Ze'ev, The Weizmann Institute of Science, Israel) was ligated into the expression vector pLK-pac (Islam et al., 1996). The E-cadherin construct has been described (Lewis et al., 1997). The human cadherin-11 cDNA was provided by Drs. S. Takashita and A. Kudo (Tokyo Institute of Technology, Japan; accession number D21254; Okazaki et al., 1994).

Antibodies and reagents: Unless otherwise stated, all reagents were from Sigma Chemical Co. (St. Louis, MO). Rabbit polyclonal antibodies (Jelly) against human E-cadherin extracellular domain (Wheelock et al., 1987) and mouse monoclonal antibodies against E-cadherin (HECD1; a kind gift of Dr. Masatoshi Takeichi, Kyoto University, Kyoto, Japan) and N-cadherin (13A9; Knudsen et al., 1995, Sacco et al., 1995) have been previously described. The mouse mAb against β -catenin (6E3) was made as previously described (Johnson et al. 1993). The mouse mAbs against cadherin-11 were kindly provided by Dr. Marion Bussemakers (University Hospital Nijmegen, The Netherlands). The diacylglycerol lipase inhibitor RHC80267 was purchased from Biomol (Plymouth Meeting, PA).

Extraction of cells: Monolayers of cells were washed with PBS at room temperature and extracted on ice with 2.5 mls/75 cm^2 flask 10 mM Tris acetate, pH 8.0, containing 0.5% Nonidet P-40 (BDH Chemicals Ltd., Poole, United Kingdom), 1 mM EDTA and 2 mM PMSF. The cells were scraped followed by vigorous pipetting for 5 min on ice. Insoluble material was removed by centrifugation at 15,000 g for 10 min at 4° C. Cell extracts were resolved on 7% SDS-PAGE as described (Lewis et al., 1994), transferred electrophoretically to nitrocellulose and immunoblotted

as described (Wheelock et al., 1987) using primary antibodies followed by ECL according to the manufacturer's protocol (Pierce Chemical Co., Rockford, IL). For the purpose of loading equal amounts of protein onto SDS-PAGE, quantification was done using the BioRad Protein Assay reagent according to the manufacturer's protocol.

Immunofluorescence and Microscopy: Cells were grown on glass coverslips, fixed with Histochoice (Amresco, Solon, OH), washed 3 times with PBS and blocked for 30 min with PBS supplemented with 10% goat serum. Coverslips were exposed to primary antibodies for 1 h, washed 3 times with PBS and exposed to species specific antibodies conjugated to FITC or Rhodamine for 1 h. Cells were viewed using a Zeiss Axiophot microscope equipped with the appropriate filters and photographed using Kodak T-MAX 3200 film. Living cells were viewed using a Zeiss Axiovert microscope and photographed using Kodak T-MAX 400 film.

***In vitro* invasion assays and motility assays:** For motility assays, 5×10^5 cells were plated in the top chamber of noncoated polyethylene terephthalate (PET) membranes (6 well insert, pore size 8 mm; Becton Dickinson, Research Triangle Park, N.C.). For *in vitro* invasion assays, 3×10^4 cells were plated in the top chamber of Matrigel coated PET membranes (24 well insert, pore size 8 mm; Becton Dickinson). In motility and invasion assays, 3T3 conditioned medium was used as a chemoattractant in the lower chamber. The cells were incubated for 24 hours and those which did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. Cells transversing the membrane were stained with Diff-Quick (Dade, Aquada, P.R.). Cells in 10 random fields of view at 100x magnification were counted and expressed as the average number of cells/field of view. Three independent experiments were done in each case. The data was represented as the average of the 3 independent experiments with the standard deviation of the average indicated. When cells were induced with dexamethasone to express a transgene the control cells were treated with the same level of dexamethasone. To inhibit fibroblast growth factor receptor signaling, cells were treated with RHC80267 (which inhibits the activity of diacylglycerol lipase) at a concentration of 10-40 $\mu\text{g/ml}$ 3T3 conditioned culture medium during the 24 hours of the assay.

3. Results

Expression of cadherins by breast cancer cells

E-cadherin has been termed a tumor suppressor, mainly because cells derived from E-cadherin-negative epithelial tumors tend to be invasive while cells derived from E-cadherin positive tumors tend not to be. In the case of cells derived from breast carcinomas, the majority of E-cadherin-negative cells are invasive (Sommers et al., 1991; Sommers et al., 1994; Pierceall et al., 1995). However, an increasing number of exceptions to this rule are becoming evident. Our laboratory has recently shown that expression of an inappropriate cadherin by an oral squamous carcinoma cell line influences expression of E-cadherin and the cellular phenotype (Islam et al., 1996). This observation led us to hypothesize that the invasiveness of breast cancer cells may be due to an increase in the expression of an inappropriate cadherin, possibly N-cadherin, rather than to a decrease in the expression of E-cadherin. To test this hypothesis, we surveyed a large number of cell lines, many of which had been previously characterized, for expression of E-cadherin and N-cadherin. The data, which are summarized in Table I, supported our notion that invasiveness is correlated with N-cadherin expression rather than lack of E-cadherin expression.

Table I

Cell Line	E-cad	N-cad	P-cad	Cad 11	β -catenin	Motility
MCF-7	+ ^{ab}	- ^a	- ^a	- ^a	+ ^{ade}	no ^{ab}
BT-20	+ ^a	- ^a	+ ^a	- ^a	+ ^a	no ^a
SUM149	+ ^a	- ^a	+ ^a	- ^a	+ ^a	no ^a
SKBr3	- ^{ab}	- ^a	- ^a	- ^a	- ^{ade}	no ^{ab}
MDA-MB-453	- ^{ab}	- ^a	- ^a	- ^a	- ^{ade}	no ^b
SUM1315	- ^a	- ^a	+ ^a	+/- ^a	+ ^a	no ^a
MDA-MB-435	- ^{ab}	+ ^a	- ^a	- ^a	+ ^{ae}	yes ^c
MDA-MB-436	- ^{ab}	+ ^a	- ^a	- ^a	+ ^{ade}	yes ^b
BT549	- ^{ab}	+ ^a	+ ^a	- ^a	+ ^{adc}	yes ^b
Hs578t	- ^{ab}	+ ^a	- ^a	- ^a	+ ^{adc}	yes ^{ab}
SUM159PT	- ^a	+ ^a	- ^a	- ^a	+ ^a	yes ^a
MDA-MB-231	- ^a	- ^a	- ^a	+ ^{af}	+ ^a	yes ^{af}

^a current study; ^b Sommers et al., 1991; ^c Frixen et al., 1991; ^d Sommers et al., 1994; ^e Pierceall et al., 1995; ^f Pishvaian et al., 1999.

Fig. 1 is an immunoblot of extracts of the cell lines presented in Table 1. Equal amounts of protein were loaded in each lane. The samples were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted for E-cadherin, N-cadherin, P-cadherin, cadherin 11 and β -catenin. Fig. 2

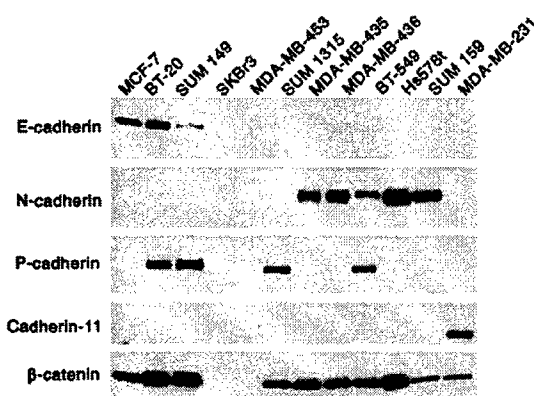


Figure 1. Cadherin and β -catenin expression in breast carcinoma cell lines. Confluent monolayers of MCF-7, BT-20, SUM149, SKBr3, MDA-MB-453, SUM1315, MDA-MB-435, MDA-MB-436, BT-549, Hs578t, SUM159PT or MDA-MB-231 were extracted with NP-40. Twenty μ g total protein from each cell extract was resolved by SDS-PAGE, transferred to nitrocellulose and blotted with antibodies against E-cadherin (HECD1), N-cadherin, P-cadherin, cadherin-11 or β -catenin.

presented phase micrographs of the living cells in order to compare the morphologies of breast cancer cells expressing the various members of the cadherin family. MCF-7 cells expressed E-cadherin, were non-invasive and presented an epithelial-like morphology. BT-20 cells expressed both E-cadherin and P-cadherin, were non-invasive and presented an epithelial-like morphology. In contrast, E-cadherin-negative cell lines did not present an epithelial morphology, but rather appeared as fibroblast-like cells with less obvious cell-cell interactions. Even the SUM149 cell line that expressed a small amount of E-cadherin, along with substantial amounts of P-cadherin, did not have the epithelial appearance typified by the MCF-7 and BT-20 cell lines. SUM1315 cells, which expressed P-cadherin along with a small amount of cadherin 11, also had a fibroblastic appearance with minimal cell-cell interactions. However, these fibroblastic, N-cadherin-negative cell lines were non-motile and were not invasive (Table I and Fig. 3). The N-cadherin-expressing cell lines all displayed a fibroblastic phenotype as typified by MDA-MB-435, MDA-MB-436 and SUM159 (Fig. 2). Cell lines that did not express any cadherin, as typified by SKBr3, displayed a fibroblastic phenotype much like the N-cadherin-positive cells, however they were less adhesive to the substratum than were cadherin-expressing cells. In addition, they tended to float in the medium upon reaching confluency and when undergoing mitosis.

A role for N-cadherin in cell motility

In this study we hypothesized that the invasive behavior of breast cancer cell lines may be due to expression of N-cadherin rather than to lack of expression of E-cadherin. To test this hypothesis we performed invasion assays on Matrigel-coated membranes and motility assays on uncoated membranes. Fig. 3 presents data from representative cell lines. The N-cadherin expressing cell lines, SUM159 and MDA-MB-435 were substantially more invasive and more motile than the E-cadherin-expressing line (MCF-7) the E/P-cadherin expressing cell lines (BT-20 and SUM149) and the P-cadherin expressing line (SUM1315). The cell line that did not express any cadherins, SKBr3, was no more motile nor invasive than were the E-cadherin expressing cell lines BT-20, MCF-7 and SUM149. Together these data suggest that, in these cells, N-cadherin acts to promote motility and invasion, rather than E-cadherin acting to suppress these activities.

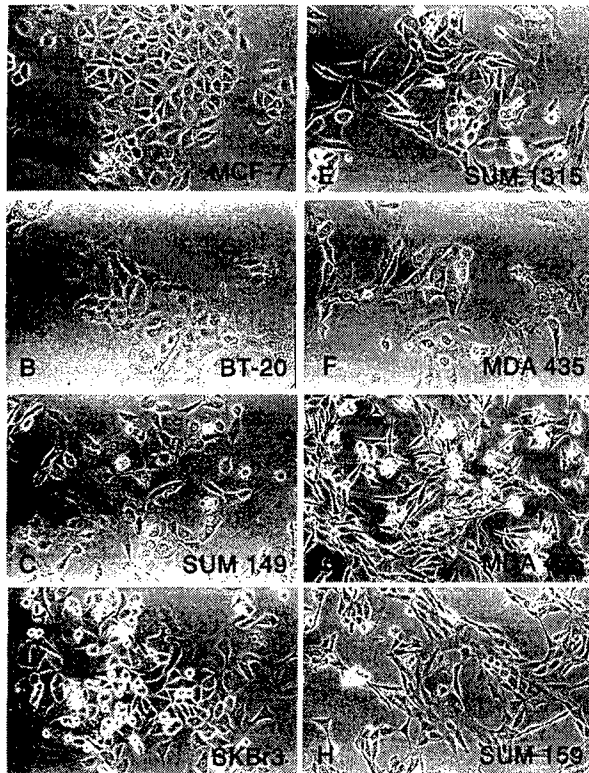


Figure 2. Morphological analysis of breast cancer cell lines. Living monolayers of A) MCF-7, B) BT-20, C) SUM149, D) SKBr3, E) SUM1315, F) MDA-MB-435, G) MDA-MB-436 or H) SUM159PT cells were photographed using an inverted Zeiss microscope at 200X magnification. Bar = 10 µm.

comparable to MDA-MB-435; however, they did not undergo a morphological change (Fig. 4 A), nor did they down-regulate the expression of E-cadherin to any significant level. Figs. 4 B and C

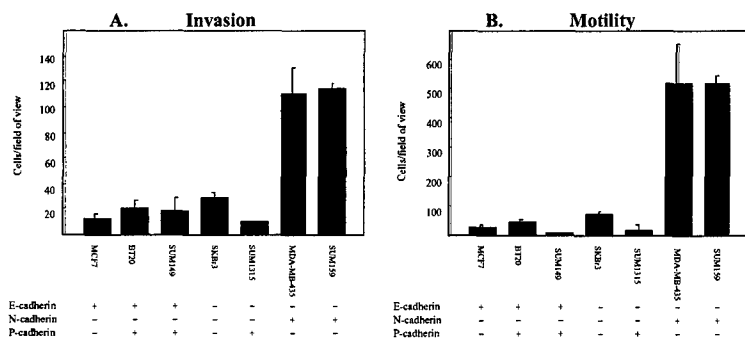


Figure 3. N-cadherin expression correlates with increased invasiveness and motility in breast carcinoma cell lines. Cells were plated on Matrigel coated or non-coated membranes for invasion assays or motility assays respectively. The cells were incubated for 24 h, and those which did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transgressing the membrane was determined by averaging 10 random fields of view at 100x magnification. The data is expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate standard deviation of the average.

Since the cell lines in this study all were derived from separate tumors and thus are likely to be descendents of different cell types, we sought to manipulate expression of specific cadherins in representative cell lines in order to determine if the invasive phenotype was due to N-cadherin or to other cellular aspects. We chose two cell lines for these studies: BT-20 which expresses E-cadherin and P-cadherin and is non-invasive, and MDA-MB-435 which expresses N-cadherin and is highly invasive. When BT-20 cells were transfected with N-cadherin (BT-20N), they expressed levels of N-cadherin that were

comparable to MDA-MB-435; however, they did not undergo a morphological change (Fig. 4 A), nor did they down-regulate the expression of E-cadherin to any significant level. Figs. 4 B and C show that E-cadherin and N-cadherin co-localize at cell-cell borders, suggesting that both cadherins are active at the cell surface. When equal amounts of protein from extracts of BT-20 and BT-20N cells were resolved by SDS-PAGE and immunoblotted for cadherin expression, it could be seen that the BT-20N cells slightly down-regulated E-cadherin, that the two cell lines expressed equal levels of P-cadherin and that the BT-20N cells expressed levels of N-cadherin that were comparable to the invasive N-cadherin-expressing cells depicted in Fig. 1. In addition, β -catenin co-immunoprecipitated equally well with either E-cadherin or N-

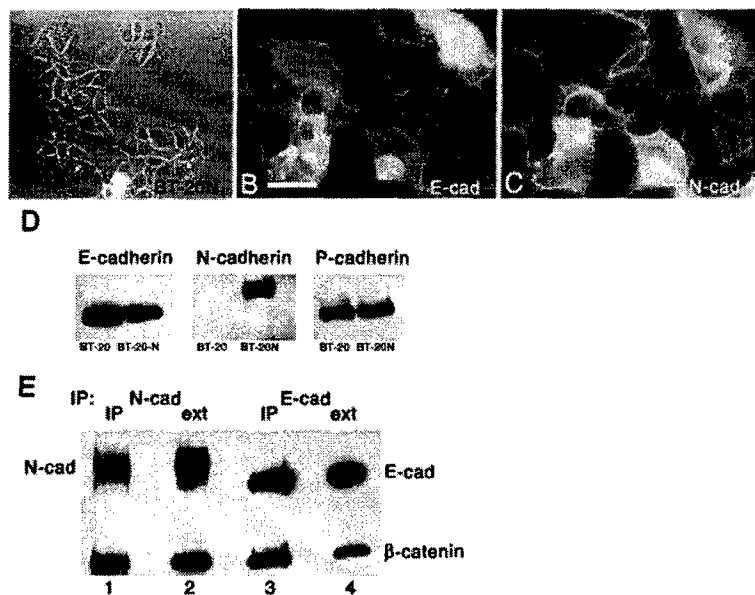


Figure 4. Expression of N-cadherin by BT-20 cells. BT-20 cells were transfected with N-cadherin (BT-20N) and expression induced with dexamethasone. A. Phase microscopy of living BT-20N cells. B and C. Cells were grown on glass coverslips and processed for co-immunofluorescence localization with antibodies against E-cadherin (Jelly) (B) and N-cadherin (C). Bar = 10 μ m. D. BT-20 and BT-20N cells were extracted with NP-40 and 20 μ g protein from each extract was resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted for E-cadherin (HECD1), N-cadherin or P-cadherin. E. Extracts of BT-20N cells were immunoprecipitated with antibodies against N-cadherin or E-cadherin (HECD1). The immunoprecipitation reactions as well as cell extracts were resolved by SDS PAGE, transferred to nitrocellulose and immunoblotted for N-cadherin and β -catenin (lanes 1 and 2) or E-cadherin (HECD1) and β -catenin (lanes 3 and 4).

cadherin in these cells (Fig. 4E). BT-20 cells were unusual in that they expressed high levels of both E-cadherin and N-cadherin and thus were an ideal cell line in which to test the hypothesis that it is the expression of N-cadherin, not the lack of E-cadherin, that promotes cell motility and invasion in some breast cancer cells. As predicted, motility and invasion rates for BT-20N were 5 to 8 fold higher than the rates for non-transfected BT-20 cells (Fig. 5). Although BT-20N cells were not as motile as the N-cadherin-expressing MDA-MB-435 cells (Fig. 5B), they were almost as invasive (Fig. 5A).

E-cadherin does not suppress motility in N-cadherin-expressing MDA-MB-435 cells

Since the BT-20N cells expressed high levels of E-cadherin and were highly motile and invasive, we had good evidence that E-cadherin did not inhibit invasion in these cells, and thus does not act as an invasion suppressor in all breast cancer cells. However, to further test this idea we transfected N-cadherin-expressing MDA-MB-435 cells with E-cadherin (MDA-MB-435E) to see if E-cadherin would decrease the invasive nature of these cells. In this experiment, we sought to obtain clones that expressed high levels of E-cadherin but still retained a significant level of N-cadherin. Fig. 6D shows the levels of expression of E-cadherin and N-cadherin in several clones. Clone 2 was chosen for subsequent studies because it expressed the highest level of E-cadherin and, in addition, showed a 2 to 3 fold reduction in N-cadherin expression compared to the parental cells. Although these cells expressed very high levels of E-cadherin, they did not display a typical epithelial morphology and closely resembled the parent cell line (compare Fig. 6A with Fig. 2). Both E-cadherin and N-cadherin were localized to regions of cell-cell contact (Figs. 6B and C). When the MDA-MB-435E cells were tested for motility and invasion, they were not significantly

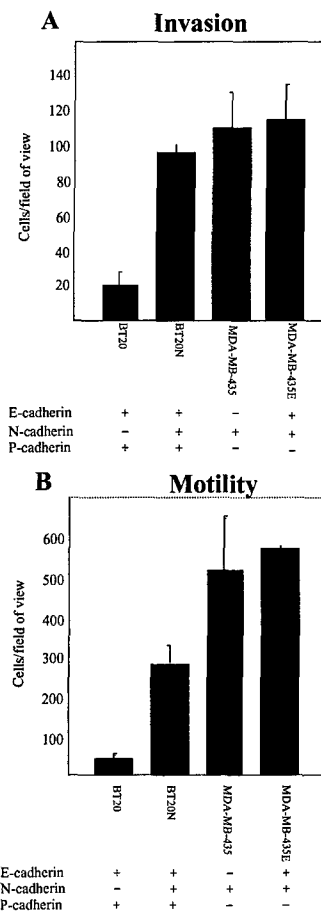


Figure 5. Cells were plated on Matrigel coated or non-coated membranes for invasion assays or motility assays, respectively. The cells were incubated for 24 h, and the number transverse the membrane is expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate standard deviation of the average.

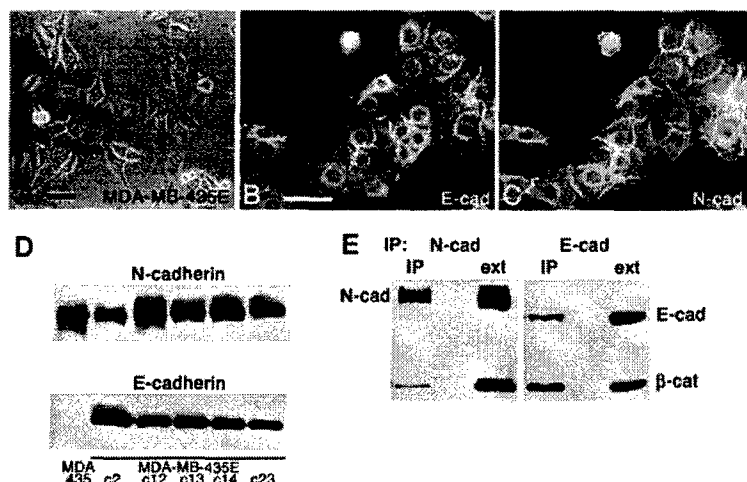


Figure 6. Expression of E-cadherin by MDA-MB-435 cells. MDA-MB-435 cells were transfected with E-cadherin (MDA-MB-435E). A. Phase microscopy. B and C. Co-immunofluorescence localization of E-cadherin (B) and N-cadherin (C). D. MDA-MB-435 and several clones of MDA-MB-435E cells were extracted, resolved by SDS PAGE and immunoblotted for E-cadherin and N-cadherin. E. Extracts of MDA-MB-435E cells were immunoprecipitated with antibodies against N-cadherin or E-cadherin. The immunoprecipitation reactions along with cell extracts were resolved by SDS-PAGE and immunoblotted for N-cadherin and β -catenin (lanes 1 and 2) or E-cadherin and β -catenin (lanes 3 and 4).

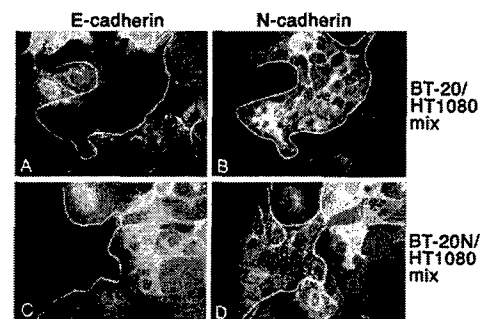


Figure 7. BT-20N cells do not mix with HT1080 cells. BT-20 or BT-20N cells were mixed with an equal number of HT1080 cells, allowed to settle on coverslips and processed for immunofluorescence for E-cadherin or N-cadherin. A and B are a mix of BT-20 and HT1080 cells stained for E-cadherin and N-cadherin respectively. The encircled cells are a group of E-cadherin-negative, N-cadherin-positive HT1080 cells. C and D are a mix of BT-20N and HT1080 cells stained for E-cadherin and N-cadherin respectively. The encircled cells are a group of E-cadherin-negative, N-cadherin-positive HT1080 cells.

different from the parental MDA-MB-435 cells (Fig. 5) even though β -catenin was associated with the transfected E-cadherin as well as the endogenous N-cadherin (Fig. 6E).

BT-20N cells effectively segregate from HT1080 fibroblasts

Hazan et al. (1997) suggested that N-cadherin-expressing breast cancer cells invade the stroma because they associate with the N-cadherin-expressing stromal cells. In our studies, we employed an *in vitro* invasion assay in which the cells invade an extracellular matrix that does not include any stromal cells. Thus, we can make the important statement that, in our studies, N-cadherin actively promotes invasion and motility. In Hazan et al. (1997), the investigators showed that N-cadherin-expressing breast cancer cells co-aggregated with N-cadherin-expressing fibroblast-like cells. Since it has been suggested that it is the entire complement of cadherins expressed by a cell that determines its ability to associate with other cells, and that even cells expressing different levels of the same cadherin can sort from one another (Steinberg and Takeichi, 1994), we sought to determine if the BT-20N cells which express N-cadherin, E-cadherin and P-cadherin would segregate from an N-cadherin-expressing fibroblast cell line, HT1080. Equal numbers of BT-20 cells and HT1080 cells or BT-20N cells and HT1080 cells were mixed together and allowed to settle on glass coverslips. They were then prepared for immunofluorescence analysis using antibodies against E-cadherin or N-cadherin. In the immunofluorescence analysis of the BT-20/HT1080 co-cultures, E-cadherin stained only the BT-20 cells and N-cadherin stained only the HT1080 cells. Figs. 7A and B show that these two cell lines effectively segregated from one another as expected. In the immunofluorescence analysis of the BT-20N/HT1080 co-cultures, antibodies against E-cadherin stained only the BT-20N cells while antibodies against N-cadherin stained both the BT-20N cells and the HT1080 cells. Figs. 7C and D show that the BT-20N cells and the HT1080 cells effectively segregated from one another even though both cell lines express N-cadherin. Thus, epithelial cells that express N-cadherin along with other cadherins have not necessarily gained the ability to intermix with stromal cells.

Cadherin-11 promotes motility in breast epithelial cells

In the course of our studies on breast tumor cell lines, we characterized one atypical line (MDA-MB-231) which did not express E-, P-, or N-cadherin but nonetheless was invasive. Since MDA-MB-231 cells expressed significant levels of β -catenin, a protein that is not stable in cadherin-

negative cells, we suspected that this cell line expressed another member of the cadherin family of

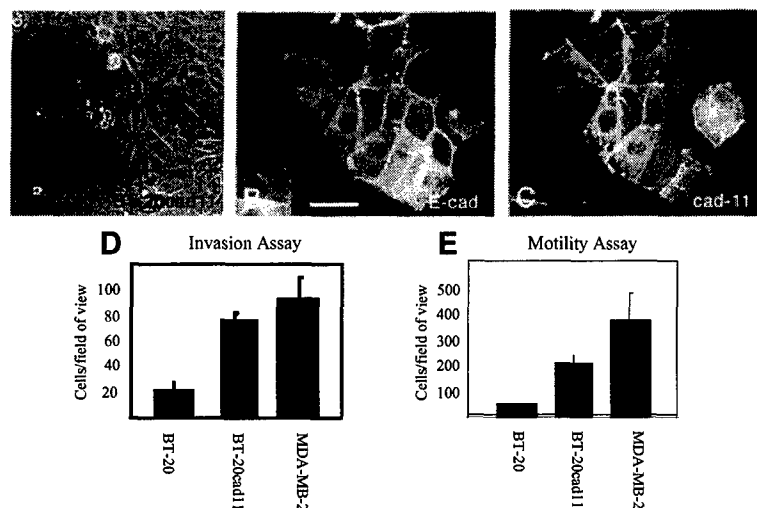


Figure 8. Exogenous expression of cadherin-11 increases invasiveness. BT-20 cells were transfected with cadherin-11 (BT-20cad11). A. Phase microscopy. B and C. Co-immunofluorescence localization of E-cadherin (B) and cadherin-11 (C). D and E. Cells were plated on Matrigel coated or non-coated membranes for invasion assays or motility assays, respectively. The number of cells transversing the membrane is the average of three independent experiments.

interactions as the cytotrophoblasts invade the uterine wall (MacCalman et al., 1996). Thus, one idea is that cadherin-11 could act in a manner similar to N-cadherin in promoting cell motility and invasion in breast cancer cells. To test this idea, we transfected cadherin-11 into BT-20 cells (BT-20Cad-11 cells). Like the BT-20N cells, BT-20Cad-11 cells retained the morphology of their parent line even though they expressed high levels of cadherin-11 at cell-cell borders (Figs. 8 A-C). As predicted, cadherin-11-expressing BT-20 cells were nearly as invasive and as motile as N-cadherin-expressing BT-20 cells (Figs. 8D and 5). Interestingly, the cadherin-11-expressing cells were not as invasive or motile as the N-cadherin-expressing cells. For example, the MDA-MB-231 cells were not as motile as the MDA-MB-435 cells (Figs. 5 and 8). More significantly, the BT-20 cells transfected with cadherin-11 did not become as motile as they did when transfected with N-cadherin. This may be due to differences between the two cadherins or differences in expression levels of the transfected cadherins. It is reasonable to speculate that the level of expression of the inappropriate cadherin is relevant since the cell line SUM1315 expresses a small amount of cadherin-11 yet is not invasive.

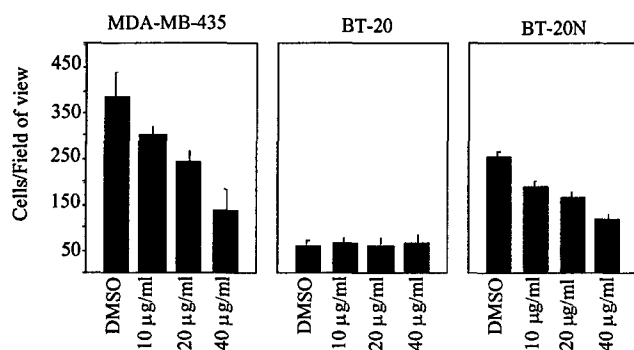
N-cadherin may promote cell motility through a fibroblast growth factor receptor signal transduction pathway

The laboratories of Frank Walsh and Patrick Doherty have shown that N-cadherin promotes neurite outgrowth from cerebellar neurons (Williams et al., 1994a). In addition, these authors showed that N-cadherin-mediated neurite extension was dependent on fibroblast growth factor (FGF) receptor signaling but was independent of ligand (Williams et al., 1994b). Walsh and Doherty thus proposed a model whereby the FGF receptor was induced to dimerize in the absence of FGF via interaction with N-cadherin (Doherty and Walsh, 1996). Dimerization of the FGF receptor results in receptor cross phosphorylation which initiates a number of signal transduction pathways. The pathway relevant to N-cadherin-dependent neurite outgrowth involves the generation of arachidonic acid from diacylglycerol by the action of diacylglycerol lipase. The Walsh and Doherty laboratories showed that the diacylglycerol lipase inhibitor RHC80267 prevented neurite extension on N-cadherin-transfected 3T3 cells thus implicating this type of FGF receptor signaling in N-cadherin-dependent neurite extension (Meiri et al., 1998). We hypothesized that the N-cadherin-mediated cell motility we observed in epithelial cells may also be acting through FGF receptor signaling. To test this hypothesis we treated MDA-MB-435 cells, BT-20 cells, and BT-20N cells with varying levels of RHC80267 to determine if it would influence the motility of these cells in the transwell assay. RHC80267 inhibited cell motility in both N-cadherin-expressing cell lines in a

proteins, possibly one that is closely related to N-cadherin. We therefore analyzed RNA from this line with degenerate PCR primers designed to amplify all cadherins and found that it expressed cadherin-11 mRNA. Expression of cadherin-11 protein was confirmed by immunoblotting data with a cadherin-11-specific monoclonal antibody, in agreement with recent data (Pishvaian et al., 1999). Like N-cadherin, cadherin-11 is expressed by some mesenchymal cells (Simonneau et al., 1995). Interestingly, cadherin-11 is expressed in some epithelial cells of the human placenta, and it has been suggested that cadherin-11 plays a role in mediating trophoblast-endometrium

dose-dependent manner (Fig. 9A). Importantly, this inhibitor had no effect on the motility of the

A



B

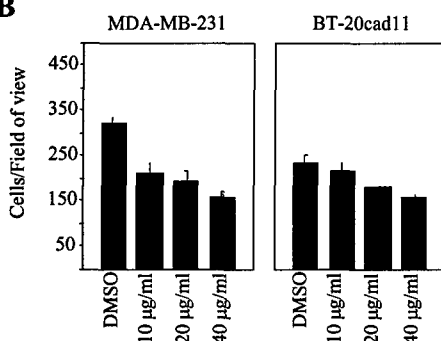


Figure 9. The diacylglycerol lipase inhibitor RHC80267 decreases motility of N-cadherin- and cadherin-11-expressing cells. Cells plated on non-coated membranes were incubated for 24 h in the presence of RHC80267 at varying concentrations and the number transversing the membrane is expressed as the number of cells/field of view and is the average of three (A) or two (B) independent experiments.

N-cadherin-negative BT-20 cells. Thus, the data are consistent with the hypothesis that N-cadherin dependent cell motility is mediated through FGF receptor signaling in a manner similar to N-cadherin-dependent neurite outgrowth. To determine if cadherin-11 and N-cadherin promote cell motility through a similar pathway, we treated MDA-MB-231 and BT-20cad11 cells with RHC80267 and compared motility rates between treated and non-treated cells (Fig. 9 B). The diacylglycerol lipase inhibitor decreased the motility of cadherin-11-expressing cells in a dose-dependent manner. Cadherin-11-expressing cells are less motile than MDA-MB-435 and the inhibitor is less effective in decreasing the motility of the cadherin-11 expressing cells, suggesting there may be some differences in the respective signal transduction pathways, possibly in growth factor receptor levels or isoforms.

4. Relationship to the approved Statement of Work

I have copied the portions of the Statement of Work from the original proposal that are appropriate for the first 2 years of this project and have indicate in green those tasks which were completed last year and in red those tasks which have been completed this year. In dark blue is our plan for the next year.

Technical objective 1. Survey cell lines and biopsies:

Task 1. Months 1-3: Surveying breast cancer cell lines for E-cadherin expression. Part of this was reported last year and part is presented in Table I and figure 1 above.

Task 2. Months 4-6: Survey E-cadherin negative cell lines for expression of N-cadherin, P-cadherin, R-cadherin and Cadherin 5. Part of this was reported last year and part is presented in Table I and figure 1 above. We are still analyzing for expression of R-cadherin and cadherin 5.

Task 3. Months 7-8. Survey frozen histological sections for expression of the cadherin identified in Task 2. We have initiated a collaborative project with a pathologist, Dr. David Rimm at Yale University, to survey frozen sections.

Task 4. Months 9-12. If we do not identify one specific cadherin in task 2 we will perform PCR using degenerate primers to identify the cadherin of interest. This is irrelevant at this point as we

have identified N-cadherin as expressed by invasive breast carcinoma cells. In addition, we have identified a new, previously unreported cadherin that shares some homology with cadherin 11. The characterization of this cadherin will be a component of the next funding period. We have further characterized this new cadherin during year 2. We have almost a full length clone now and will continue to characterize it during the next year.

At the end of year 1, we expect to have identified an inappropriate cadherin that is expressed in breast tumors. We accomplished our goals for year one and reported this last year.

Task 5. Months 12-18. Prepare antibodies against the newly identified cadherin (X-cadherin) if necessary. A fusion protein has been made in *E. coli*, is being injected into mice. We hope to have an antibody within the next year.

Technical objective 2. Determine if the expression of inappropriate cadherins contributes to tumorigenesis.

Task 6. Months 12-18: Obtain normal breast cell lines from the Michigan Tissue Bank. Transfect them with X-cadherin and observe the morphology of the transfected cells. We have transfected BT-20 cells with N-cadherin and reported the results of this experiment above.

Task 7. Months 12-18: Transfect the tumor cells that express X-cadherin with antisense X-cadherin and observe the morphology of the transfected cells. We have determined that this is not a feasible experiment. The anti-sense has been transfected and is not effective in down-regulating N-cadherin.

Task 8. Months 18-22: Assay the normal cells, the transfected normal cells from task 6, the tumor cells, the transfected tumor cells from task 7 for motility and invasive characteristics. This has been accomplished and is reported above.

Technical objective 3. Explore the mechanisms that regulate the expression of cadherins in breast tumor cells.

Task 9. Months 22-24: Transform normal breast epithelial cells with ras and determine the levels of expression of E-cadherin and the inappropriate cadherin(s) found in technical objectives 1 and 2 above. Our ideas on this topic have changed since the submission of the original grant. We are pursuing the idea that transformation to the tumorigenic phenotype may be regulated by the FGF receptor. This is discussed above and preliminary data are presented above. We will continue to analyze the involvement of FGFR signaling during the next year.

Task 10. Month 24: Survey the cell lines that express X-cadherin for expression of erbB-2, EGF receptor and p53. Determine if there is a correlation between any of these markers and expression of X-cadherin or down-regulation of E-cadherin. Our ideas on this topic have changed since the submission of the original grant. We are pursuing the idea that transformation to the tumorigenic phenotype may be regulated by the FGF receptor. This is discussed above and preliminary data are presented above. We will continue to analyze the involvement of FGFR signaling during the next year.

Task 11. Months 25-31: Transfect normal breast cells with markers identified in task 10 to determine if overexpression of this marker results in down-regulation of E-cadherin or increased expression of X-cadherin. During the next funding period, we will activate FGFR in breast cancer cell lines to determine if there is a relationship between invasion and FGFR.

Task 12. Months 24-30: Treat normal breast epithelial cells with estrogen and progesterone to determine if these hormones have an effect on the expression of E-cadherin or other cadherins. Treat normal breast epithelial cells with TGF β and other TGF β family members to determine if these hormones have an effect on the expression of E-cadherin or other cadherins. This will be initiated during the next funding period.

Task 13. Months 30-36: Analyze the data from task 12 and propose a mechanism for regulation of cadherin expression that can be further explored during the final 6 months of this project.

Key Research Accomplishments

- ◆ In this study we explored the possibility that expression of non-epithelial cadherins may be correlated with increased cellular motility and invasion in human breast cancer cells.
- ◆ We present data showing that N-cadherin promotes cell motility and invasion in breast cancer cells.
- ◆ We showed that decreased expression of E-cadherin does not necessarily correlate with invasion in breast cancer cells.
- ◆ We showed that N-cadherin expression correlates both with invasion and motility in breast cancer cells and likely plays a direct role in promoting motility.
- ◆ We showed that forced expression of E-cadherin in invasive, N-cadherin positive cells does not reduce their motility or invasive capacity.
- ◆ We showed that forced expression of N-cadherin in non-invasive, E-cadherin-positive cells produces an invasive cell even though these cells continue to express high levels of E-cadherin.
- ◆ We present data suggesting that N-cadherin-dependent motility may be mediated by fibroblast growth factor receptor signaling.
- ◆ We showed that cadherin 11 acts in a manner similar to N-cadherin.

Reportable Outcomes

- ◆ A paper has been published in the Journal of Cell Biology. The citation is: Nieman, M.T., Prudoff, R.S., Johnson, K.R. and Wheelock, M.J. 1999. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. J. Cell Biol. 147:631-643.
- ◆ This work was presented as an invited talk at the Biological Structure and Gene Expression Gordon Conference in 1999.
- ◆ This work was presented as a platform talk at the American Society for Cell Biology Meeting in 1999.
- ◆ This work was presented as an invited talk at the International Bat-Sheva de Rothschild Seminar Weizmann Institute, Rehovot, Israel
- ◆ This work was presented at the Era of Hope DOD Breast Cancer Meeting in 2000.
- ◆ Marvin Nieman graduated with a Ph.D. in biology based on work supported on this project.
- ◆ Several cell lines were generated by transfecting cadherins into breast cancer cells.
- ◆ Dr. Marvin Nieman was granted a position at the University of Michigan as a postdoctoral fellow based on his work as a Ph.D. student in my laboratory.
- ◆ Ryan Prudoff was accepted into medical school in part due to his work on this project.

Conclusions

Our laboratory previously showed that expression of different cadherin family members by squamous epithelial cells markedly effected morphology (Islam et al., 1996), i.e., when oral squamous epithelial cells expressed N-cadherin, they converted to a fibroblastic phenotype concurrent with decreased cell-cell adhesion. Thus, when we turned our attention to breast cancer cells for the present study, we were interested not only in the expression of various cadherins by these cells, but also in whether these cadherins influenced the morphology of the cells. We were not surprised to find that breast cancer cells endogenously expressing N-cadherin displayed a fibroblastic phenotype with tenuous cell-cell contacts, while breast cancer cells endogenously expressing E-cadherin displayed a typical epithelial morphology. We were, however, surprised to find that transfection of N-cadherin into the E-cadherin-expressing BT-20 breast cancer cell line had no effect on morphology even though it had a dramatic effect on cell behavior. Equally surprising was the fact that forced expression of E-cadherin had no effect on the morphology of the fibroblastic N-cadherin-expressing MDA-MB-435 cells. Thus, the breast cancer cell lines examined in this study behaved very differently from the oral squamous epithelial lines that we previously characterized. In our previous study, E-cadherin-expressing oral squamous epithelial cells attained a fibroblastic morphology when they were transfected with N-cadherin. Interestingly, the oral squamous epithelial cells down-regulated E-cadherin when they were forced to express N-cadherin, suggesting an inverse relationship between these cadherins. In contrast, the breast cancer cells continued to express their endogenous cadherin when transfected with a different cadherin. The continued expression of endogenous cadherin may account for the lack of morphological change in the transfectants. Thus, the breast cancer cells differ from the oral squamous epithelial cells in two very important ways: 1. The oral squamous epithelial cells appear to co-regulate cadherins in an inverse manner while these cadherins are independently regulated in breast cancer cells; and 2. Expression of E-cadherin by the oral squamous epithelial cells is sufficient to convert a fibroblast-like cell to an epithelial morphology while epithelial morphology in the breast cancer cells appears to depend on other factors in addition to E-cadherin.

In the present study, we have demonstrated that N-cadherin (or cadherin-11) expression in human breast carcinoma cells promotes an invasive phenotype. By transfecting the non-invasive BT-20 cells with these non-epithelial cadherins, we have provided evidence for a direct role of these cadherins in cell motility and invasion. Previous studies have correlated the expression of N-cadherin or cadherin-11 with invasion in breast cancer cells. However, in this study we took the important next step and used transfection studies to show that a previously non-invasive cell could be converted to an invasive cell by expression of N-cadherin or cadherin-11. The BT-20 breast cancer cell line provided an important tool for these studies since they did not down-regulate E-cadherin when forced to express N-cadherin. Thus, we can conclude that, even in cells expressing high levels of E-cadherin, N-cadherin (or cadherin-11) can promote motility, suggesting that, in this regard, both N-cadherin and cadherin-11 are "dominant" over E-cadherin. A study by Sommers et al. (1994) supports this idea. These authors showed that transfection of E-cadherin into the E-cadherin-negative breast cancer cell lines BT549 and HS578 did not decrease the invasive capacity of these cells. These authors suggested that the transfected E-cadherin was not functional; however, these authors were unaware of the fact that the BT549 and HS578 cell lines express N-cadherin.

A previous study utilizing MDA-MB-435 cells showed that transfection of E-cadherin into these cells reduced their capacity to form tumors when injected into the foot pads of nude mice (Meiners et al., 1998). In contrast to our study, these authors showed that E-cadherin-transfected clones of MDA-MB-435 cells underwent a morphological change upon E-cadherin expression. In addition, they showed that E-cadherin-transfected clones were more tumorigenic in their assay than were the parental cells. One difference in the study of Meiners et al. (1998) and ours is that they did not assay for N-cadherin expression in their E-cadherin-positive clones of MDA-MB-435 transfectants. Our study clearly demonstrates that N-cadherin influences the behavior of the cells and that cells retaining N-cadherin do not undergo a morphological or behavioral change upon

expression of E-cadherin. Thus one possible explanation for the difference between these two studies is that the cells in the Meiners study did not express N-cadherin. The point of our study was to determine if N-cadherin was capable of influencing the behavior of epithelial cells even if they expressed E-cadherin, thus we were particularly careful to select cell lines that retained N-cadherin expression after transfection with E-cadherin (Fig. 6).

One puzzling aspect of cell lines derived from metastatic tumors is that they often express E-cadherin and appear to be relatively normal epithelial cells. A possibility suggested by our results is that these cells upregulated the expression of N-cadherin during the process of metastasis. Our results suggest that expression of N-cadherin would confer on these cells the capacity to invade even though they continued to express E-cadherin. In this regard, expression of an inappropriate cadherin like N-cadherin (or other related cadherins) may be a better gauge of the clinical state of a tumor than is decreased expression of E-cadherin.

Some of the E-cadherin-negative breast cancer cells expressed endogenous P-cadherin. These cells had a fibroblastic morphology similar to that of the N-cadherin-expressing cells; however, they were not invasive, suggesting that P-cadherin confers upon breast cancer cells characteristics different from those conferred by either E-cadherin or N-cadherin. P-cadherin is expressed in the myoepithelial cells surrounding the luminal epithelial cells of the mammary gland, and Radice et al. (1997) recently showed that P-cadherin deficient mice develop age-dependent hyperplasia and dysplasia of the mammary epithelium and suggested that P-cadherin may play a role in maintaining the normal phenotype of breast epithelial cells. One possibility is that the P-cadherin-expressing tumor cells were derived from the myoepithelium rather than from the true epithelium.

E-cadherin has been termed an invasion suppressor because transfection of this protein into some E-cadherin-negative invasive carcinoma cells resulted in decreased invasive capacity. Our prediction is that at least some of these cell lines expressed a cadherin, like N-cadherin or cadherin-11, and over-expression of E-cadherin resulted in down-regulation of the endogenous cadherin as we saw with the oral squamous epithelial cells. Thus, we hypothesize that the invasion suppressor role of E-cadherin arises in part from its ability to decrease the level of N-cadherin in certain but not all tumors. In the present study, cell lines that did not express any classical cadherins, as evidenced by lack of β -catenin protein, as well as lack of detectable cadherin, were not invasive. Our hypothesis that loss of E-cadherin alone does not necessarily increase invasive capacity in breast carcinoma cells is supported by the observation that function blocking antibodies against E-cadherin did not confer a motile, invasive phenotype on MCF-7 cells, a breast cancer cell line that is E-cadherin-positive and N-cadherin-negative (Sommers et al., 1991). The current study suggests that, in some carcinoma cells, expression of N-cadherin, or a similar cadherin such as cadherin-11, may actually be necessary for increased motility and invasion. A recent clinical study suggested that inactivation of E-cadherin is an early event in the progression of lobular breast carcinomas (Vos et al., 1997). We might suggest here that a subsequent event would be activation of the expression of an inappropriate cadherin such as N-cadherin or cadherin-11.

Understanding the mechanism by which N-cadherin promotes motility in epithelial cells is important if we are to develop treatments that will decrease the invasiveness of tumor cells. A number of studies have shown that epithelial cells can be induced to scatter in response to growth factors such as hepatocyte growth factor and members of the fibroblast growth factor, epidermal growth factor and transforming growth factor families (Vallés et al., 1990; Blay and Brown, 1985; Geimer and Bade, 1991; Miettinen et al., 1994; Behrens et al., 1991; Gherardi and Stoker 1991; Rosen et al., 1991; Savagner et al., 1994; Savagner et al., 1997). Walsh, Doherty and co-workers have established through extensive studies on FGF receptor and cell adhesion molecules that N-cadherin and the FGF receptor cooperate to induce neurite outgrowth in cerebellar neurons (reviewed in Doherty and Walsh, 1996; Walsh and Doherty, 1997). These authors have proposed a scheme for activation of the kinase activity of the FGF receptor through cis interactions with N-cadherin via an HAV domain in the FGF receptor and an HAV interaction domain in the fourth extracellular domain of N-cadherin (Doherty and Walsh, 1996). In addition, it has been proposed

that the cadherins form lateral dimers in the plane of the membrane (Shapiro et al., 1995; Takeda et al., 1999), which could result in dimerization of the FGF receptor and subsequent activation of the signal transduction pathway. We based the studies presented herein on the model presented by Walsh and Doherty and proposed that interaction of N-cadherin with the FGF receptor in N-cadherin-expressing epithelial cells may result in increased motility similar to that seen by treating epithelial cells with growth factors. To test this hypothesis, we interfered with the N-cadherin-dependent FGF receptor signal transduction pathway proposed by Walsh and Doherty by inhibiting a downstream enzyme, diacylglycerol lipase. We showed that inhibiting diacylglycerol lipase decreased motility of N-cadherin-expressing cells in a dose-dependent manner while having no effect on the motility of N-cadherin-negative cells. Thus, our data strongly support the notion that N-cadherin promotes motility in breast cancer cells by activating growth factor receptor signal transduction pathways.

At first glance it might seem unlikely that expression of an additional cell adhesion molecule would confer a motile and invasive phenotype upon an epithelial cell. However, motile cells such as fibroblasts and myoblasts express N-cadherin (Knudsen et al., 1995; Huttenlocher et al., 1998) and a switch from E-cadherin to N-cadherin occurs in the chick embryo when epiblast cells ingress through the primitive streak to form the mesoderm (Edelman et al., 1983; Hatta and Takeichi 1986). Another interesting cadherin switch occurs during establishment of the human placenta where fetal cytotrophoblast cells invade the vasculature of the uterus. During this invasive process, the cytotrophoblast cells down-regulate the expression of E-cadherin and up-regulate vascular/endothelial (VE) cadherin (Zhou et al., 1997). Thus, it is feasible to suggest that increased expression of a non-epithelial cell cadherin such as N-cadherin could increase the invasive potential of tumor cells. Ongoing studies in our laboratory are designed to determine how N-cadherin differs from E-cadherin in its ability to induce cell motility. We hypothesize that E-cadherin does not have the ability to interact with the relevant growth factor receptors, and we are preparing chimeric molecules between E-cadherin and N-cadherin to test this hypothesis.

An important message from the present studies is that cadherins may not function identically in different cell types. The fact that cadherins may act differently in different cell types is particularly evident when comparing the current study with earlier studies showing that mouse L cells or S180 fibroblasts attained an epithelial morphology when transfected with either E-cadherin or N-cadherin (Nagafuchi et al., 1987; Hatta et al., 1988; Matsuzaki et al., 1990). It will be important in future studies to consider the cellular make up as well as the complement of cadherin family members when interpreting data on cellular morphology and behavior.

References

- Aberle, H., S. Butz, J. Stappert, H. Weissig, R. Kemler, and H. Hoschuetzky. 1994. Assembly of the cadherin-catenin complex in vitro with recombinant proteins. *J. Cell Sci.* 107:3655-3663.
- Aono, S., S. Nakagawa, A.B. Reynolds, and M. Takeichi. 1999. p120(ctn) acts as an inhibitory regulator of cadherin function in colon carcinoma cells. *J. Cell Biol.* 145:551-562.
- Behrens, J., K.M. Weidner, U.H. Frixen, J.H. Schipper, M. Sachs, N. Arakaki, Y. Daikuhara, and W. Birchmeier. 1991. The role of E-cadherin and scatter factor in tumor invasion and cell motility. *EXS.* 59:109-126.
- Behrens, J., M. Mareel, F.M. van Roy, and W. Birchmeier. 1989. Dissecting tumor cell invasion: Epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. *J. Cell Biol.* 108:2435-2447.
- Berx, G., A.M. Cleton-Jansen, F. Nollet, W.J.F. de Leeuw, M.J. van de Vijver, M.J. Cornelisse, and F. van Roy. 1995. E-cadherin is a tumor/invasion suppressor gene mutated in human lobular breast cancers. *EMBO J.* 14:6107-6115.

Berx, G., A.M. Cleton-Jansen, K. Strumane, W.J. de Leeuw, F. Nollet, F. van Roy, and C. Cornelisse. 1996. E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. *Oncogene*. 13:1919-1925.

Blay, J., and K.D. Brown. 1985. Epidermal growth factor promotes the chemotactic migration of cultured rat intestinal epithelial cells. *J. Cell. Physiol.* 124:107-112.

Bringuier, P.P., R. Umbas, H.E. Schaafsma, H.F. Karthaus, F.M. Debruyne, and J.A. Schalken. 1993. Decreased E-cadherin immunoreactivity correlates with poor survival in patients with bladder tumors. *Cancer Res.* 53:3241-3245.

Chen, H., N. Paradies, M. Fedor-Chaiken, and R. Brackenbury. 1997. E-cadherin mediates adhesion and suppresses cell motility via distinct mechanisms. *J. Cell Sci.* 110:345-356.

Chen, W.C., and B. Öbrink. 1991. Cell-cell contacts mediated by E-cadherin (uvomorulin) restrict invasive behavior of L-cells. *J. Cell Biol.* 114:319-327.

Daniel, J.M., and A.B. Reynolds. 1995. The tyrosine kinase substrate p120^{cas} binds directly to E-cadherin but not to the adenomatous polyposis coli protein or α -catenin. *Mol. Cell. Biol.* 15:4819-4824.

Doherty, P., and F.S. Walsh. 1996. CAM-FGF receptor interactions: A model for axonal growth. *Molec. Cell. Neurosci.* 8:99-111.

Dorudi, S., J.P. Sheffield, R. Poulsom, J.M. Northover, and I.R. Hart. 1993. E-cadherin expression in colorectal cancer. An immunocytochemical and in situ hybridization study. *Am. J. Pathol.* 142:981-986.

Edelman, G.M., W.J. Gallin, A. Delougee, B.A. Cunningham, and J.P. Thiery. 1983. Early epochal maps of two different cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 80:4384-4388.

Frixen, U.H., J. Behrens, M. Sachs, G. Eberle, B. Voss, A. Warda, D. Lochner, and W. Birchmeier. 1991. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J. Cell Biol.* 113:173-185.

Gamello, C., J. Palacios, A. Suarez, A. Pizarro, P. Novarro, M. Quintanilla, and A. Cano. 1993. Correlation of E-cadherin expression with differentiation grade and histological grade in breast carcinoma. *Am. J. Pathol.* 142:987-993.

Geimer, P., and E.G. Bade. 1991. The epidermal growth factor-induced migration of rat liver epithelial cells is associated with a transient inhibition of DNA synthesis. *J. Cell Sci.* 100:349-355.

Gherardi, E., and M. Stoker. 1991. Hepatocyte growth factor-scatter factor: mitogen, motogen, and met. *Cancer Cells.* 3:227-232.

Guriec, N., L. Marcellin, B. Gairard, H. Calderoli, A. Wilk, R. Renaud, J.P. Bergerat, and F. Oberling. 1996. E-cadherin mRNA expression in breast carcinomas correlates with overall and disease-free survival. *Invasion Metastasis.* 16:19-26.

Hamaguchi, M., N. Matsuyoshi, Y. Ohnishi, B. Gotoh, M. Takeichi, and Y. Nagai. 1993. p60v-src causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO J.* 12:307-314.

Hatta, K., and M. Takeichi. 1986. Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. *Nature*. 320:447-449.

Hatta, K., A. Nose, A. Nagafuchi, and M. Takeichi. 1988. Cloning and expression of cDNA encoding a neural calcium-dependent cell adhesion molecule: its identity in the cadherin gene family. *J. Cell Biol.* 106:873-881.

Hazan, R.B., L. Kang, B.P. Whooley, and P.I. Borgen. 1997. N-cadherin promotes adhesion between invasive breast cancer cells and the stroma. *Cell Adhes. Commun.* 4:399-411.

Huttenlocher, A., M. Lakonishok, M. Kinder, S. Wu, T. Truong, K.A. Knudsen, and A. F. Horwitz. Integrin and cadherin synergy regulates contact inhibition of migration and motile activity. 1998. *J. Cell Biol.* 141:515-526.

Islam, S., T.E. Carey, G.T. Wolf, M.J. Wheelock, and K.R. Johnson. 1996. Expression of N-cadherin by human squamous carcinoma cells induces a scattered fibroblastic phenotype with disrupted cell-cell adhesion. *J. Cell Biol.* 135:1643-1654.

Johnson, K.R., J.E. Lewis, D. Li, J. Wahl, A.P. Soler, K.A. Knudsen, and M.J. Wheelock. 1993. P- and E-cadherin are in separate complexes in cells expressing both cadherins. *Exp. Cell Res.* 207:252-260.

Knudsen, K.A., A.P. Soler, K.R. Johnson, and M.J. Wheelock. 1995. Interaction of α -actinin with the cadherin/catenin cell-cell adhesion complex via α -catenin. *J. Cell Biol.* 130:67-77.

Lewis, J. E., P.J. Jensen, K.R. Johnson, and M.J. Wheelock. 1994. E-cadherin mediates adherens junction organization through protein kinase C. *J. Cell Sci.* 107:3615-3621.

Lewis, J.E., J.K. Wahl III, K.M. Sass, P.J. Jensen, K.R. Johnson, and M.J. Wheelock. 1997. Cross-talk between adherens junctions and desmosomes depends on plakoglobin. *J. Cell Biol.* 136:919-934.

Luo, J., D.M. Lubaroff, and M.J.C. Hendrix. 1999. Suppression of prostate cancer invasive potential and matrix metalloproteinase activity by E-cadherin transfection. *Cancer Res.* 59:3552-3556.

MacCalman, C.D., E.E. Furth, A. Omigbodun, M. Bronner, C. Coutifaris, and J.F. Strauss III, 1996. Regulated expression of cadherin-11 in human epithelial cells: a role for cadherin-11 in trophoblast-endometrium interactions? *Dev. Dyn.* 206:201-211.

Mayer, B., J.P. Johnson, F. Leidl, K.W. Jauch, M.M. Heiss, F.W. Schildberg, W. Birchmeier, and I. Funke. 1993. E-cadherin expression in primary and metastatic gastric cancer: down-regulation correlates with cellular dedifferentiation and glandular disintegration. *Cancer Res.* 53:1690-1695.

Matsuyoshi, N., M. Hamaguchi, S. Tanaguchi, A. Nagafuchi, S. Tsukita, and M. Takeichi. 1992. Cadherin-mediated cell-cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J. Cell Biol.* 118:703-714.

Matsuzaki, F., R.M. Mege, S.H. Jaffe, D.R. Friedlander, W.J. Gallin, J.I. Goldberg, B.A. Cunningham, and G.M. Edelman. 1990. cDNAs of cell adhesion molecules of different specificity induce changes in cell shape and border formation in cultured S180 cells. *J. Cell Biol.* 110:1239-1252.

- Meiners, S., V. Brinkmann, H. Naundorf, and W. Birchmeier. 1998. Role of morphogenetic factors in metastasis of mammary carcinoma cells. *Oncogene*. 16:9-20.
- Meiri, K.F., J.L. Saffell, F.S. Walsh, and P. Doherty. 1998. Neurite outgrowth stimulated by neural cell adhesion molecules requires growth-associated protein-43 (GAP-43) function and is associated with GAP-43 phosphorylation in growth cones. *J. Neurosci.* 15:10429-10437.
- Miettinen, P.J., R. Ebner, A.R. Lopez, and R. Derynck. 1994. TGF- β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *J. Cell Biol.* 127:2021-2036.
- Moll, R., M. Mitze, U.H. Frixen, and W. Birchmeier. 1993. Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. *Am. J. Pathol.* 143:1731-1742.
- Nagafuchi, A., Y. Shirayoshi, K. Okazaki, K. Yasuda, and M. Takeichi. 1987. Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature*. 329:341-343.
- Nagafuchi, A., S. Ishihara, and S. Tsukita. 1994. The roles of catenins in the cadherin-mediated cell adhesion: Functional analysis of E-cadherin- α -catenin fusion molecules. *J. Cell Biol.* 127:235-245.
- Nieset, J.E., A. R. Redfield, F. Jin, K.A. Knudsen, K. R. Johnson, and M.J. Wheelock. 1997. Characterization of the interactions of α -catenin with α -actinin and β -catenin/plakoglobin. *J. Cell Sci.* 110:1013-1022.
- Oka, H., H. Shiozaki, K. Kobayashi, M. Inoue, H. Tahara, T. Kobayashi, Y. Takatsuka, N. Matsuyoshi, S. Hirano, M. Takeichi, and T. Mori. 1993. Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. *Cancer Res.* 53:1696-1701.
- Okazaki, M., S. Takeshita, S. Kawai, R. Kikuno, A. Tsujimura, A. Kudo, and E. Amann. 1994. Molecular cloning and characterization of OB-cadherin, a new member of cadherin family expressed in osteoblasts. *J. Biol. Chem.* 269:12092-12098.
- Ohkubo, T., and M. Ozawa. 1999. p120(ctn) binds to the membrane-proximal region of the E-cadherin cytoplasmic domain and is involved in modulation of adhesion activity. *J. Biol. Chem.* 274:21409-21415.
- Pierceall, W.E., A.S. Woodard, J.S. Morrow, D. Rimm, and E.R. Fearon. 1995. Frequent alterations in E-cadherin and α - and β -catenin expression in human breast cancer cell lines. *Oncogene*. 11:1319-1326.
- Pishvaian, M.J., C.M. Feltes, P. Thompson, M.J. Bussemakers, J.A. Schalken, and S.W. Byers. 1999. Cadherin-11 is expressed in invasive breast cancer cell lines. *Cancer Res.* 15:947-952.
- Radice, G.L., M.C. Ferreira-Cornwell, S.D. Robinson, H. Rayburn, L.A. Chodosh, M. Takeichi, R.O. Hynes. 1997. Precocious mammary gland development in P-cadherin-deficient mice. *J. Cell Biol.* 139:1025-1032.
- Rasbridge, S.A., C.E. Gillett, S.A. Sampson, F.S. Walsh, and R.R. Millis. 1993. Epithelial (E-) and placental (P-) cadherin cell adhesion molecule expression in breast carcinoma. *J. Pathol.* 169:245-250.

- Reynolds, A.B., J. Daniel, P.D. McCrea, M.J. Wheelock, J. Wu, and Z. Zhang. 1994. Identification of a new catenin: the tyrosine kinase substrate p120^{cas} associates with E-cadherin complexes. *Mol. Cell. Biol.* 14:8333-8342.
- Rimm, D. L., E.R. Koslov, P. Kebriaei, C.D. Cianci, and J.S. Morrow. 1995. α_1 (E)-catenin is an actin-binding and-bundling protein mediating the attachment of F-actin to the membrane adhesion complex. *Proc. Natl. Acad. Sci. USA.* 92:8813-8817.
- Rosen, E.M., J. Knesel, and I.D. Goldberg. 1991. Scatter factor and its relationship to hepatocyte growth factor. *Cell Growth Differ.* 2:603-607.
- Sacco, P. A., T.M. McGranahan, M.J. Wheelock, and K.R. Johnson. 1995. Identification of plakoglobin domains required for association with N-cadherin and α -catenin. *J. Biol. Chem.* 270: 20201-20206.
- Savagner, P., A.M. Vallés, J. Jouanneau, K.M. Yamada, and J.P. Thiery. 1994. Alternative splicing in fibroblast growth factor receptor 2 is associated with induced epithelial-mesenchymal transition in rat bladder carcinoma cells. *Mol. Biol. Cell.* 5:851-862.
- Savagner, P., K.M. Yamada, and J.P. Thiery. 1997. The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. *J. Cell Biol.* 137:1403-1419.
- Schipper, J.H., U.H. Frixen, J. Behrens, A. Unger, K. Jahnke, and W. Birchmeier. 1991. E-cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. *Cancer Res.* 51:6328-6337.
- Shapiro, L., A.M. Fannon, P.D. Kwong, A. Thompson, M.S. Lehman, G. Grubel, J.F. Legrand, J. Als-Nielsen, D.R. Colman, and W.A. Hendrickson. 1995. Structural basis of cell-cell adhesion by cadherins. *Nature* 374:327-337.
- Shibamoto, S., M. Hayakawa, K. Takeuchi, T. Hori, K. Miyazawa, N. Kitamura, K.R. Johnson, M.J. Wheelock, N. Matsuyoshi, M. Takeichi, and F. Ito. 1995. Association of p120, a tyrosine kinase substrate, with E-cadherin/catenin complexes. *J. Cell Biol.* 128:949-957.
- Simonneau, L., M. Kitagawa, S. Suzuki, and J.P. Thiery. 1995. Cadherin 11 expression marks the mesenchymal phenotype: towards new functions for cadherins?. *Cell Adhes. Commun.* 3:115-130.
- Sommers, C.L., E.W. Thompson, J.A. Torri, R. Kemler, E.P. Gelmann, and S.W. Byers. 1991. Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacities. *Cell Growth Differ.* 2:365-372.
- Sommers, C.L., E.P. Gelmann, R. Kemler, P. Cowin, and S.W. Byers. 1994. Alterations in β -catenin phosphorylation and plakoglobin expression in human breast cancer cells. *Cancer Res.* 54:3544-3552.
- Stappert, J., and R. Kemler. 1994. A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. *Cell Adhes. Commun.* 2:319-327.
- Steinberg, M.S., and M. Takeichi. 1994. Experimental specification of cell sorting, tissue spreading, and specific spatial patterning by quantitative differences in cadherin expression. *Proc. Natl. Acad. Sci. USA* 91:206-209.

- Takeda, H., Y. Shimoyama, A. Nagafuchi, and S. Hirohashi. 1999. E-cadherin functions as a cis-dimer at the cell-cell adhesive interface in vivo. *Nat Struct Biol.* 6:310-312.
- Umbas, R., W.B. Isaacs, P.P. Bringuier, H.E. Schaafsma, H.F. Karthaus, G.O. Oosterhof, F.M. Debruyne, and J.A. Schalken. 1994. Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. *Cancer Res.* 54:3929-3933.
- Valles, A.M., B. Boyer, J. Badet, G.C. Tucker, D. Barritault, J.P. and Thiery. 1990. Acidic fibroblast growth factor is a modulator of epithelial plasticity in a rat bladder carcinoma cell line. *Proc. Natl. Acad. Sci. USA.* 87:1124-1128.
- Vos, C.B., A.M. Cleton-Jansen, G. Berx, W.J. de Leeuw, N.T. ter Haar, F. van Roy, C.J. Cornelisse, J.L. Peterse, and M.J. van de Vijver. 1997. E-cadherin inactivation in lobular carcinoma in situ of the breast: an early event in tumorigenesis. *Br. J. Cancer.* 76:1131-1133.
- Walsh, F.S., and P. Doherty. 1997. Neural cell adhesion molecules of the immunoglobulin super family: Role in axonal growth and guidance. *Annu. Rev. Cell Biol.* 13:425-456.
- Watabe-Uchida, M. N. Uchida, Y. Imamura, A. Nagafuchi, K. Fujimoto, T. Uemura, S. Vermeulen, F. van Roy, E.D. Adamson, and M. Takeichi. 1998. α -Catenin-vinculin interaction functions to organize the apical junctional complex in epithelial cells. *J. Cell Biol.* 142:847-857.
- Wheelock, M.J., C.A. Buck, K.B. Bechtol, and C.H. Damsky. 1987. Soluble 80-kd fragment of cell-CAM 120/80 disrupts cell-cell adhesion. *J. Cell Biochem.* 34:187-202.
- Wheelock, M. J., K.A. Knudsen, and K.R. Johnson. 1996. Membrane-cytoskeleton interactions with cadherin cell adhesion proteins: roles of catenins as linker proteins. *Curr. Top. Membr.* 43:169-185.
- Williams, E.J., F.S. Walsh, and P. Doherty. 1994a. Tyrosine kinase inhibitors can differentially inhibit integrin-dependent and CAM-stimulated neurite outgrowth. *J. Cell Biol.* 124:1029-1037.
- Williams, E.J., J. Furness, F.S. Walsh, and P. Doherty. 1994b. Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. *Neuron.* 13:583-594.
- Zhou Y., S.J. Fisher, M. Janatpour, O. Genbacev, E. Dejana, M.J. Wheelock, and C.H. Damsky. 1997. Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J. Clin. Invest.* 99:2139-2151.

Appendix

1. Abstract from an invited talk at the Biological Structure and Gene Expression Gordon Conference in 1999.
2. Abstract from an invited talk at the American Society for Cell Biology Meeting in 1999.
3. Abstract from an invited talk at the International Bat-Sheva de Rothschild Seminar Weizmann Institute, Rehovot, Israel
4. Abstract from a poster presented at the Era of Hope DOD Breast Cancer Meeting in 2000.
5. Copy of : Nieman et al. 1999. N-cadherin promotes motility in human breast cancer cells regardless of their E-cadherin expression. *J. Cell Biol.* 147:631-643.

1. Abstract from an invited talk at the Biological Structure and Gene Expression Gordon Conference in 1999.

Decreased expression of E-cadherin has been correlated with increased tumor cell invasion. In some systems, inappropriate expression of a non-epithelial cadherin by an epithelial cell has been shown to down-regulate E-cadherin expression and to contribute to a scattered phenotype. In this study we show that N-cadherin correlates with increased cell motility and invasion in human breast cancer cells. The points we make are:

- A. that N-cadherin expression correlates both with invasion and motility in breast cancer cells and likely plays a direct role in promoting motility
- B. that decreased expression of E-cadherin does not necessarily correlate with invasion in breast cancer cells
- C. that forced expression of E-cadherin in invasive, N-cadherin positive cells does not reduce their motility or invasive capacity
- D. that forced expression of N-cadherin in non-invasive, E-cadherin-positive cells produces an invasive cell even though these cells continue to express high levels of E-cadherin
- E. that increased invasiveness of N-cadherin-expressing breast carcinoma cells is likely not due to interactions with stromal cells.

2. Abstract from an invited talk at the American Society for Cell Biology Meeting in 1999.

E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell-cell adhesion and plays a role in maintaining the normal phenotype of human breast epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a non-epithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to down-regulate E-cadherin expression and to increase the invasive potential of the cell. In this study we explored the possibility that expression of non-epithelial cadherins may be correlated with increased invasion in human breast cancer cells. We showed that up-regulation of inappropriate cadherins, rather than down-regulation of E-cadherin, correlates with increased motility and invasion. In most cases breast epithelial cells with reduced E-cadherin expression have turned on the expression of an inappropriate cadherin. However, we found examples of cells with reduced E-cadherin that did not express an inappropriate cadherin. In these cases, the cells were non-motile and non-invasive.

3. Abstract from an invited talk at the International Bat-Sheva de Rothschild Seminar Weizmann Institute, Rehovot, Israel

E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell-cell adhesion and plays a role in maintaining the normal phenotype of human breast epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a non-epithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to down-regulate E-cadherin expression and to contribute to a scattered phenotype. In this study we explored the possibility that expression of non-epithelial cadherins may be correlated with increased cellular motility and invasion in human breast cancer cells. We present data showing that N-cadherin promotes cell motility and invasion in breast cancer cells; that decreased expression of E-cadherin does not necessarily correlate with invasion in breast cancer cells; that N-cadherin expression correlates both with invasion and motility in breast cancer cells and likely plays a direct role in promoting motility; that forced expression of E-cadherin in invasive, N-cadherin positive cells does not reduce their motility or invasive capacity; and that forced expression of N-cadherin in non-invasive, E-cadherin-positive cells produces an invasive cell even though these cells continue to express high levels of E-cadherin.

4. Abstract from a poster presented at the Era of Hope DOD Breast Cancer Meeting in 2000.

E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell-cell adhesion and plays a role in maintaining the normal phenotype of epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a non-epithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to down-regulate E-cadherin expression and to contribute to a scattered phenotype. In this study we explored the possibility that expression of non-epithelial cadherins may be correlated with increased motility and invasion in breast cancer cells. We show that N-cadherin promotes motility and invasion; that decreased expression of E-cadherin does not necessarily correlate with motility or invasion; that N-cadherin expression correlates both with invasion and motility and likely plays a direct role in promoting motility; that forced expression of E-cadherin in invasive, N-cadherin positive cells does not reduce their motility or invasive capacity; that forced expression of N-cadherin in non-invasive, E-cadherin-positive cells produces an invasive cell even though these cells continue to express high levels of E-cadherin; that N-cadherin-dependent motility may be mediated by fibroblast growth factor receptor signaling; and that cadherin 11 promotes epithelial cell motility in a manner similar to N-cadherin.

N-Cadherin Promotes Motility in Human Breast Cancer Cells Regardless of their E-Cadherin Expression

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Abstract. E-cadherin is a transmembrane glycoprotein that mediates calcium-dependent, homotypic cell-cell adhesion and plays a role in maintaining the normal phenotype of epithelial cells. Decreased expression of E-cadherin has been correlated with increased invasiveness of breast cancer. In other systems, inappropriate expression of a nonepithelial cadherin, such as N-cadherin, by an epithelial cell has been shown to downregulate E-cadherin expression and to contribute to a scattered phenotype. In this study, we explored the possibility that expression of nonepithelial cadherins may be correlated with increased motility and invasion in breast cancer cells. We show that N-cadherin promotes motility and invasion; that decreased expression of E-cadherin does not necessarily correlate with motil-

ity or invasion; that N-cadherin expression correlates both with invasion and motility, and likely plays a direct role in promoting motility; that forced expression of E-cadherin in invasive, N-cadherin-positive cells does not reduce their motility or invasive capacity; that forced expression of N-cadherin in noninvasive, E-cadherin-positive cells produces an invasive cell, even though these cells continue to express high levels of E-cadherin; that N-cadherin-dependent motility may be mediated by FGF receptor signaling; and that cadherin-11 promotes epithelial cell motility in a manner similar to N-cadherin.

Key words: N-cadherin • E-cadherin • breast cancer • motility • fibroblast growth factor receptor

CADHERINS constitute a family of transmembrane glycoproteins that mediate calcium-dependent homotypic cell-cell adhesion and play an important role in the maintenance of normal tissue architecture. The cadherin intracellular domain interacts with several proteins, collectively called catenins, that link cadherins to the actin cytoskeleton (reviewed in Wheelock et al., 1996). This linkage is required for full cadherin adhesive activity. Either β -catenin or plakoglobin binds directly to the cadherin and to α -catenin, whereas α -catenin links directly and indirectly to actin (Aberle et al., 1994; Nagafuchi et al., 1994; Stappert and Kemler, 1994; Knudsen et al., 1995; Rimm et al., 1995; Nieset et al., 1997; Watabe-Uchida et al., 1998). Their ability to simultaneously self-associate and link to the actin cytoskeleton enables cadherins to mediate both the cell recognition required for cell sorting and the strong cell-cell adhesion needed to form tissues.

In addition to their structural role in the adherens junction, catenins are thought to regulate the adhesive activity of cadherins. For example, phosphorylation of β -catenin in Src transformed cells may contribute to the nonadhesive phenotype of these cells (Matsuyoshi et al., 1992;

Hamaguchi et al., 1993). In addition, p120^{cas}, originally identified as a Src substrate and subsequently shown to bind to the cytoplasmic domain of cadherins, has been suggested to play a role in regulating the adhesive activity of cadherins (Reynolds et al., 1994; Daniel and Reynolds, 1995; Shibamoto et al., 1995; Aono et al., 1999; Ohkubo and Ozawa, 1999).

Numerous studies have demonstrated the importance of the E-cadherin/catenin complex in maintaining the normal phenotype of epithelial cells. Early studies showed that inhibiting E-cadherin activity with function-perturbing antibodies altered the morphology of MDCK cells and conferred upon them the ability to invade both collagen gels and embryonic chicken heart tissue (Behrens et al., 1989; Chen and Öbrink, 1991). In addition, invasive, fibroblast-like carcinoma cells could be converted to a noninvasive phenotype by transfection with a cDNA encoding E-cadherin (Frixen et al., 1991). Moreover, E-cadherin expression is downregulated or lost in epithelial tumors from various tissues, including stomach, colon, head and neck, bladder, prostate, and breast (Schipper et al., 1991; Bringuier et al., 1993; Dorudi et al., 1993; Mayer et al., 1993; Oka et al., 1993; Umbas et al., 1994).

It has been suggested that alterations in cadherin function may be a critical step in the development of breast cancers. A survey of 18 cell lines derived from breast carci-

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nomas showed that ten lines failed to express detectable levels of E-cadherin, and two other lines failed to express α -catenin (Pierceall et al., 1995). Other studies have identified breast tumor cell lines with mutations in the E-cadherin gene (Berx et al., 1995), or with changes in the levels of expression or in the phosphorylation state of β -catenin or plakoglobin (Sommers et al., 1994). Surveys of breast cancer tissue make an equally compelling case for the involvement of E-cadherin in the formation or progression of breast tumors, and clinical studies have shown that loss of E-cadherin correlates with metastatic disease and poor prognosis (Gamello et al., 1993; Moll et al., 1993; Oka et al., 1993; Rasbridge et al., 1993; Berx et al., 1996; Guriec et al., 1996).

In vitro studies support the role of E-cadherin as an invasion suppressor gene. For example, forced expression of E-cadherin in rat astrocytoma cells suppressed motility (Chen et al., 1997). Likewise, transfection of invasive E-cadherin-negative breast or prostate cell lines with mouse E-cadherin resulted in cells that were less invasive in vitro assays (Frixen et al., 1991; Luo et al., 1999). When treated with function blocking E-cadherin antibodies, the transfected cells returned to an invasive phenotype, thus implicating E-cadherin as an invasion suppressor (Frixen et al., 1991).

Although a number of studies with breast carcinoma cell lines have shown that loss of E-cadherin generally results in an invasive phenotype, important exceptions have been reported. In one study, two E-cadherin-negative cell lines were shown to be noninvasive (Sommers et al., 1991). These authors suggested that in order for E-cadherin-negative cells to be invasive, they must also express vimentin.

In another study, Sommers et al. (1994) showed that transfection of E-cadherin into the invasive breast cancer cell lines, BT549 and HS578t, altered neither the morphology nor the invasive behavior of these cells. These authors speculated that the transfected E-cadherin may not be fully functional in these cells, due to altered posttranslational modification of the cadherin-associated proteins β -catenin, α -catenin, or plakoglobin.

It has been suggested that, unlike E-cadherin, N-cadherin may promote motility and invasion in carcinoma cells. For example, Hazan et al. (1997) reported that expression of N-cadherin by breast carcinoma cells correlated with invasion, and suggested that invasion was potentiated by N-cadherin-mediated interactions between the breast cancer cells and stromal cells. A study conducted in our laboratory suggested that N-cadherin may play a more direct role in the process of invasion and may actually promote invasion by inducing a scattered phenotype when expressed by oral squamous cell carcinoma-derived cells (Islam et al., 1996). In this study, forced expression of N-cadherin resulted in downregulation of endogenous E- and P-cadherins, making it impossible to separate the motility-promoting effects of N-cadherin from the motility-suppressing activity of E-cadherin. In contrast, it has been suggested that N-cadherin promotes contact inhibition in normal skeletal muscle myoblasts and, in so doing, inhibits migration upon contact, but does not suppress motility in subconfluent cells (Huttenlocher et al., 1998).

Thus, the information in the literature concerning the role cadherins may play in tumor cell invasion is inconclusive and even contradictory, prompting us to revisit the question using new reagents generated by our laboratory to examine both previously studied and newly derived breast cancer cell lines. The data presented in this paper indicate: decreased expression of E-cadherin does not necessarily correlate with invasion in breast cancer cells; N-cadherin expression correlates both with invasion and motility in breast cancer cells, and likely plays a direct role in promoting motility; forced expression of E-cadherin in invasive, N-cadherin-positive cells does not reduce their motility or invasive capacity; forced expression of N-cadherin in noninvasive, E-cadherin-positive cells produces an invasive cell, even though these cells continue to express high levels of E-cadherin; the data suggest that N-cadherin-mediated cell motility may be stimulated by FGF receptor signaling; and other cadherins, such as cadherin-11, may promote motility in epithelial cells in a manner similar to N-cadherin.

Materials and Methods

Cells

Breast carcinoma cell lines were obtained from American Type Culture Collection (ATCC) and maintained in DME with 10% FBS (SKBr3, MDA-MB-435, MDA-MB-436, BT-549, and HS578t) or MEM with 10% FBS (MDA-MB-453 and BT-20). The cell lines MCF-7 and MDA-MB-231 were obtained from Dr. Mary J.C. Hendrix (University of Iowa, Iowa City, IA) and maintained in DME with 10% FBS. The cell lines SUM 159PT and SUM 149 were kindly provided by Dr. Steve Ethier and generated by the University of Michigan Human Breast Cell/Tissue Bank and Data Base. They were maintained in Ham's F-12 with 5% FBS supplemented with insulin (5 mg/ml) and hydrocortisone (1 mg/ml). The cell line SUM 1315 was obtained from the same source and maintained in Ham's F-12 with 5% FBS supplemented with insulin (5 mg/ml) and EGF (10 ng/ml). HT1080 cells were obtained from ATCC and maintained in DME with 10% FBS.

Transfections

To transfect MDA-MB-435 with E-cadherin, the calcium phosphate transfection kit (Stratagene) was used, according to manufacturer's protocol. For electroporations (BT-20 cells), 10^6 cells were washed with PBS and resuspended in electroporation buffer (120 mM KCl, 0.15 mM CaCl_2 , 10 mM K_2HPO_4 , 10 mM KH_2PO_4 , 25 mM Hepes, 2 mM EGTA, 5 mM MgCl_2) supplemented with 2 mM ATP and 5 mM glutathione. After a 5 min incubation on ice, the cells were electroporated at 500 μF and 380 V in a Bio-Rad gene pulser. Cells were immediately plated in a 100-mm dish in complete medium. Floating cells were removed and fresh medium was added 24 h after electroporation; puromycin was added to the culture for selection of clones 48 h after electroporation.

Clones and Vectors

For transfection of N-cadherin, a restriction fragment containing nucleotides 442-3362 (GenBank/EMBL/DBJ accession number S42303; a kind gift of Dr. Avri Ben-Ze'ev, The Weizmann Institute of Science, Israel) was ligated into the expression vector pLX-pac (Islam et al., 1996). The E-cadherin construct has been described previously (Lewis et al., 1997). The human cadherin-11 cDNA was provided by Drs. S. Takashita and A. Kudo (Tokyo Institute of Technology, Japan; accession number D21254; Okazaki et al., 1994).

Antibodies and Reagents

Unless otherwise stated, all reagents were from Sigma Chemical Co. Rabbit polyclonal antibodies (Jelly) against human E-cadherin extracellular domain (Wheelock et al., 1987), and mouse mAbs against E-cadherin

(HECD1; a kind gift of Dr. Masatoshi Takeichi, Kyoto University, Kyoto, Japan) and N-cadherin (13A9; Knudsen et al., 1995; Sacco et al., 1995), have been described previously. The mouse mAb against β -catenin (6E3) was made as described by Johnson et al. (1993). The mouse mAbs against cadherin-11 were kindly provided by Dr. Marion Bussemakers (University Hospital Nijmegen, The Netherlands). The diacylglycerol lipase inhibitor, RHC80267, was purchased from BIOMOL.

Extraction of Cells

Monolayers of cells were washed with PBS at room temperature and extracted on ice with 2.5 ml/75 cm² flask 10 mM Tris acetate, pH 8.0, containing 0.5% NP-40 (BDH Chemicals Ltd.), 1 mM EDTA, and 2 mM PMSF. The cells were scraped, followed by vigorous pipetting for 5 min on ice. Insoluble material was removed by centrifugation at 15,000 g for 10 min at 4°C. Cell extracts were resolved on 7% SDS-PAGE as described (Lewis et al., 1994), transferred electrophoretically to nitrocellulose, and immunoblotted as described (Wheelock et al., 1987) using primary antibodies followed by ECL, according to the manufacturer's protocol (Pierce Chemical Co.). For the purpose of loading equal amounts of protein onto SDS-PAGE, quantification was done using the BioRad protein assay reagent according to the manufacturer's protocol.

Immunofluorescence and Microscopy

Cells were grown on glass coverslips, fixed with Histochoice (Amresco), washed three times with PBS, and blocked for 30 min with PBS supplemented with 10% goat serum. Coverslips were exposed to primary antibodies for 1 h, washed three times with PBS, and exposed to species-specific antibodies conjugated to FITC or rhodamine for 1 h. Cells were viewed using a Zeiss Axiophot microscope equipped with the appropriate filters, and photographed using Kodak T-MAX 3200 film. Living cells were viewed using a Zeiss Axiovert microscope and photographed using Kodak T-MAX 400 film.

In Vitro Invasion Assays and Motility Assays

For motility assays, 5×10^5 cells were plated in the top chamber of non-coated polyethylene terephthalate (PET) membranes (6-well insert, pore size 8 mm; Becton Dickinson). For in vitro invasion assays, 3×10^4 cells were plated in the top chamber of Matrigel-coated PET membranes (24-well insert, pore size 8 mm; Becton Dickinson). In motility and invasion assays, 3T3 conditioned medium was used as a chemoattractant in the lower chamber. The cells were incubated for 24 h and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. Cells transverse the membrane were stained with Diff-Quick (Dade). Cells in ten random fields of view at 100 \times were counted and expressed as the average number of cells/field of view. Three independent experiments were done in each case. The data were represented as the average of the three independent experiments with the SD of the average indicated. When cells were induced with dexamethasone to express a transgene, the control cells were treated with the same level of dexamethasone. To inhibit FGF receptor signaling, cells were treated with RHC80267 (which inhibits the activity of diacylglycerol lipase) at a concentration of 10–40 μ g/ml 3T3 conditioned culture medium during the 24 h of the assay.

Results

Expression of Cadherins by Breast Cancer Cells

E-cadherin has been termed a tumor suppressor, mainly because cells derived from E-cadherin-negative epithelial tumors tend to be invasive, whereas cells derived from E-cadherin-positive tumors tend not to be. In the case of cells derived from breast carcinomas, the majority of E-cadherin-negative cells are invasive (Sommers et al., 1991, 1994; Pierceall et al., 1995). However, an increasing number of exceptions to this rule are becoming evident. Our laboratory has recently shown that expression of an inappropriate cadherin by an oral squamous carcinoma cell line influences expression of E-cadherin and the cellu-

Table I. Cadherin Expression in Breast Carcinoma Cell Lines

Cell line	E-cadherin	N-cadherin	P-cadherin	Cadherin-11	β -Catenin	Motility
MCF-7	+* [‡]	—*	—*	—*	+* [‡]	No* [‡]
BT-20	+*	—*	+*	—*	+*	No*
SUM149	+*	—*	+*	—*	+*	No*
SKBr3	—* [‡]	—*	—*	—*	—* [‡]	No* [‡]
MDA-MB-453	—* [‡]	—*	—*	—*	—* [‡]	No [‡]
SUM1315	—*	—*	+*	±*	+*	No*
MDA-MB-435	—* [‡]	+*	—*	—*	+* [‡]	Yes [§]
MDA-MB-436	—* [‡]	+*	—*	—*	+* [‡]	Yes [‡]
BT549	—* [‡]	+*	+*	—*	+* [‡]	Yes [‡]
Hs578t	—* [‡]	+*	—*	—*	+* [‡]	Yes* [‡]
SUM159PT	—*	+*	—*	—*	+*	Yes*
MDA-MB-231	—*	—*	—*	+* [‡]	+*	Yes* [‡]

*Current study; [‡]Sommers et al., 1991; [§]Frixen et al., 1991; [‡]Sommers et al., 1994; [§]Pierceall et al., 1995; [†]Pishvaian et al., 1999.

lar phenotype (Islam et al., 1996). This observation led us to hypothesize that the invasiveness of some breast cancer cells may be due to an increase in the expression of an inappropriate cadherin, possibly N-cadherin, rather than to a decrease in the expression of E-cadherin. To test this hypothesis, we surveyed a large number of cell lines, many of which had been characterized previously, for expression of E- and N-cadherin. The data, which are summarized in Table I, supported our notion that invasiveness is correlated with N-cadherin expression, rather than lack of E-cadherin expression.

Fig. 1 is an immunoblot of extracts of the cell lines presented in Table I. Equal amounts of protein were loaded in each lane. The samples were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E-, N-, or P-cadherin, cadherin-11, and β -catenin. Fig. 2 presents phase micrographs of the living cells to compare the

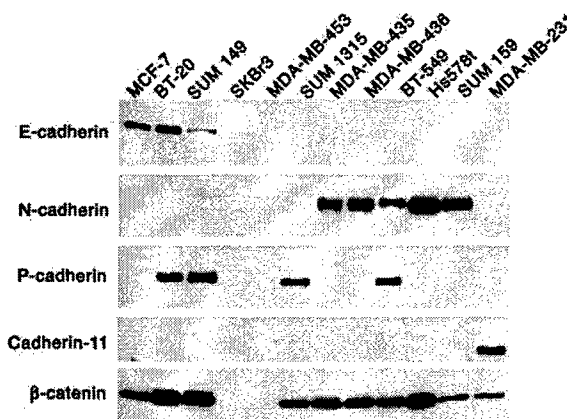


Figure 1. Cadherin and β -catenin expression in breast carcinoma cell lines. Confluent monolayers of MCF-7, BT-20, SUM 149, SKBr3, MDA-MB-453, SUM 1315, MDA-MB-435, MDA-MB-436, BT-549, Hs578t, SUM 159PT, or MDA-MB-231 were extracted with NP-40. 20 μ g total protein from each cell extract was resolved by SDS-PAGE, transferred to nitrocellulose, and blotted with antibodies against E-cadherin (HECD1), N-cadherin, P-cadherin, cadherin-11, or β -catenin.

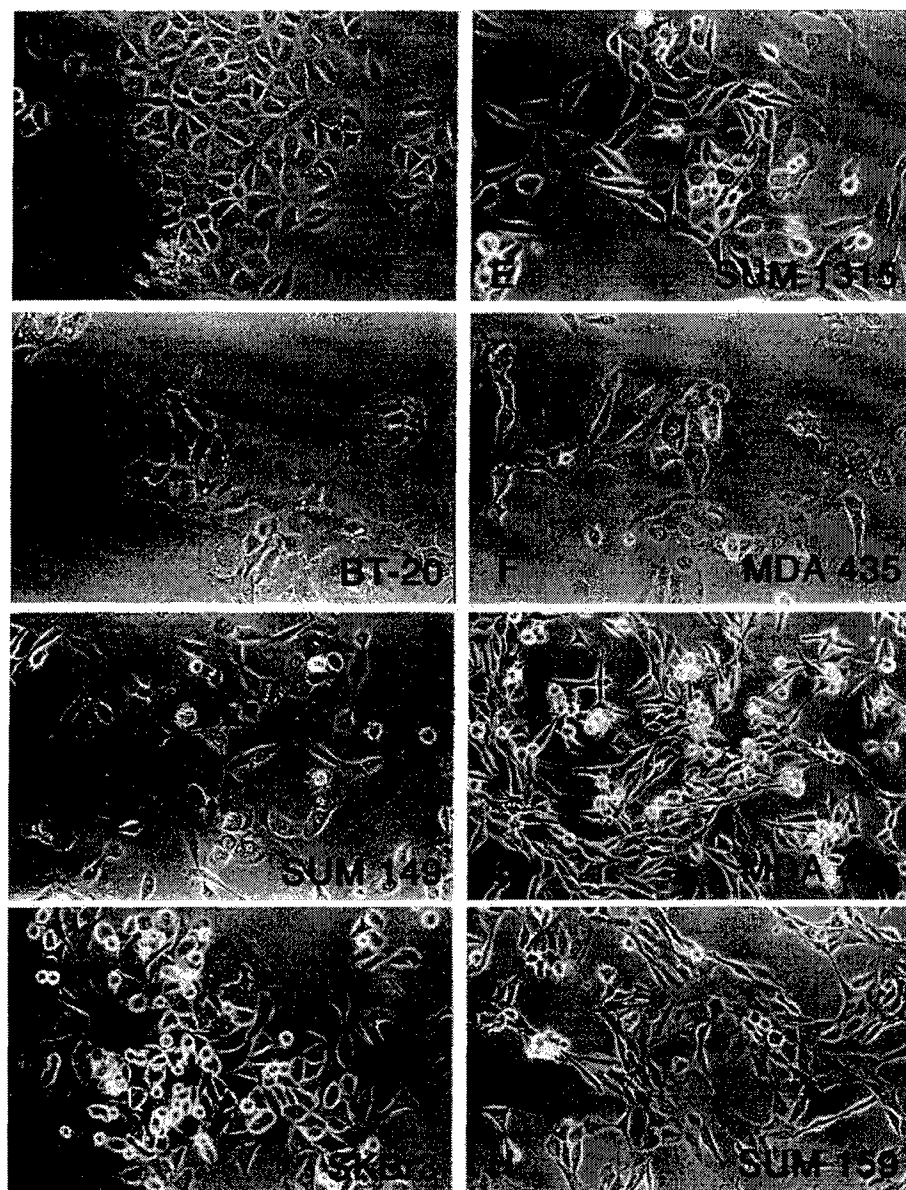


Figure 2. Morphological analysis of breast cancer cell lines. Living monolayers of MCF-7 (A), BT-20 (B), SUM 149 (C), SKBr3 (D), SUM 1315 (E), MDA-MB-435 (F), MDA-MB-436 (G), or SUM 159PT (H) cells were photographed using an inverted Zeiss microscope at 200 \times . Bar, 10 μ m.

morphologies of breast cancer cells expressing the various members of the cadherin family. MCF-7 cells expressed E-cadherin, had low invasion rates, and presented an epithelial-like morphology. BT-20 cells expressed both E- and P-cadherin, had low invasion rates, and presented an epithelial-like morphology. In contrast, E-cadherin-negative cell lines did not present an epithelial morphology, but rather appeared as fibroblast-like cells with less obvious cell-cell interactions. Even the SUM149 cell line that expressed a small amount of E-cadherin, along with substantial amounts of P-cadherin, did not have the epithelial appearance typified by the MCF-7 and BT-20 cell lines. SUM1315 cells, which expressed P-cadherin, along with a small amount of cadherin-11, also had a fibroblastic appearance with minimal cell-cell interactions. However,

these fibroblastic, N-cadherin-negative cell lines had low motility and invasion rates (Table I and Fig. 3). The N-cadherin-expressing cell lines all displayed a fibroblastic phenotype, as typified by MDA-MB-435, MDA-MB-436, and SUM159 (Fig. 2). Cell lines that did not express any cadherin, as typified by SKBr3, displayed a fibroblastic phenotype much like the N-cadherin-positive cells, however, they were less adhesive to the substratum than were cadherin-expressing cells. In addition, they tended to float in the medium upon reaching confluency and when undergoing mitosis.

A Role for N-Cadherin in Cell Motility

In this study, we hypothesized that the invasive behavior

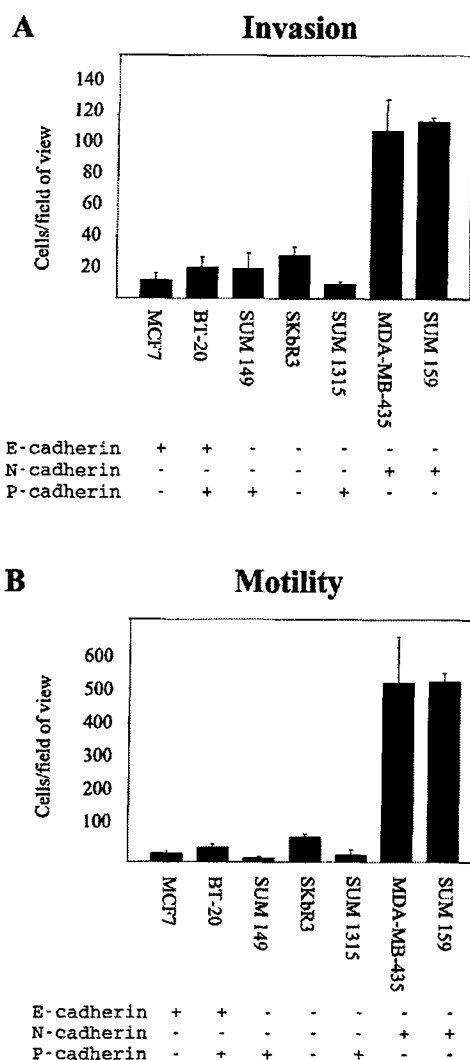


Figure 3. N-cadherin expression correlates with increased invasiveness and motility in breast carcinoma cell lines. Cells were plated on Matrigel-coated or noncoated membranes for invasion assays or motility assays, respectively. The cells were incubated for 24 h, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transverse the membrane was determined by averaging ten random fields of view at 100 \times . The data are expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate SD of the average.

of some breast cancer cell lines may be due to expression of N-cadherin, rather than to lack of expression of E-cadherin. To test this hypothesis, we performed invasion assays on Matrigel-coated membranes and motility assays on uncoated membranes. Fig. 3 presents data from representative cell lines. The N-cadherin-expressing cell lines, SUM159 and MDA-MB-435, were substantially more invasive and more motile than the E-cadherin-expressing line (MCF-7), the E/P-cadherin-expressing cell lines (BT-20

and SUM149), and the P-cadherin-expressing line (SUM 1315). The cell line that did not express any cadherins, SKBr3, was no more motile nor invasive than were the E-cadherin-expressing cell lines BT-20, MCF-7, and SUM 149. Together, these data suggest that, in these cells, N-cadherin acts to promote motility and invasion, rather than E-cadherin acting to suppress these activities.

Since the cell lines in this study were derived from separate tumors and, thus, are likely to be descendents of different cell types, we sought to manipulate expression of specific cadherins in representative cell lines to determine if the invasive phenotype was due to N-cadherin or to other cellular aspects. We chose two cell lines for these studies: BT-20, which expresses E- and P-cadherin and has a low rate of invasion, and MDA-MB-435, which expresses N-cadherin and is highly invasive. When BT-20 cells were transfected with N-cadherin (BT-20N), they expressed levels of N-cadherin that were comparable to MDA-MB-435; however, they did not undergo a morphological change (compare Fig. 2 B with Fig. 4 A), nor did they downregulate the expression of E-cadherin to any significant level. Fig. 4, B and C, show that E- and N-cadherin colocalized at cell-cell borders, suggesting that both cadherins are active at the cell surface. When equal amounts of protein from extracts of BT-20 and BT-20N cells were resolved by SDS-PAGE and immunoblotted for cadherin expression, it could be seen that the BT-20N cells slightly downregulated E-cadherin, that the two cell lines expressed equal levels of P-cadherin, and that the BT-20N cells expressed levels of N-cadherin that were comparable to the invasive N-cadherin-expressing cells depicted in Fig. 1. In addition, β -catenin coimmunoprecipitated equally well with either E- or N-cadherin in these cells (Fig. 4 E). BT-20 cells were unusual in that they expressed high levels of both E- and N-cadherin and, thus, were an ideal cell line in which to test the hypothesis that it is the expression of N-cadherin, not the lack of E-cadherin, that promotes cell motility and invasion in some breast cancer cells. As predicted, motility and invasion rates for BT-20N were five- to eightfold higher than the rates for nontransfected BT-20 cells (Fig. 5). Although BT-20N cells were not as motile as the N-cadherin-expressing MDA-MB-435 cells (Fig. 5 B), they were almost as invasive (Fig. 5 A).

E-Cadherin Does Not Suppress Motility in N-Cadherin-expressing MDA-MB-435 Cells

Since the BT-20N cells expressed high levels of E-cadherin, and were highly motile and invasive, we had good evidence that E-cadherin did not inhibit invasion in these cells and, thus, does not act as an invasion suppressor in all breast cancer cells. However, to further test this idea, we transfected N-cadherin-expressing MDA-MB-435 cells with E-cadherin (MDA-MB-435E) to see if E-cadherin would decrease the invasive nature of these cells. In this experiment, we sought to obtain clones that expressed high levels of E-cadherin, but still retained a significant level of N-cadherin. Fig. 6 D shows the levels of expression of E- and N-cadherin in several clones. Clone 2 was chosen for subsequent studies because it expressed the highest level of E-cadherin and, in addition, showed a two- to threefold reduction in N-cadherin expression, compared

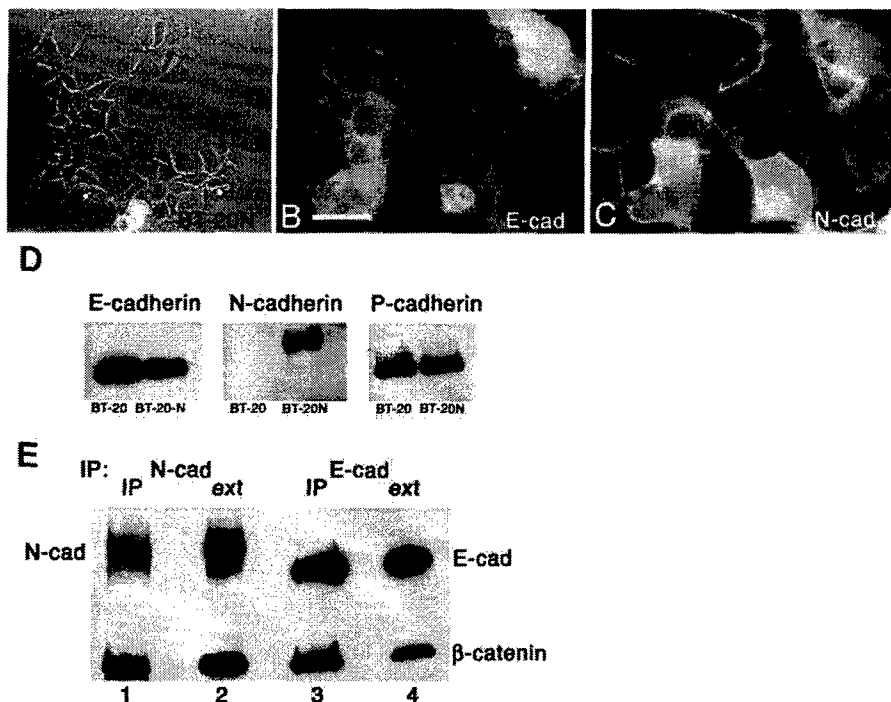


Figure 4. Expression of N-cadherin by BT-20 cells. BT-20 cells were transfected with N-cadherin (BT-20N) and expression induced with dexamethasone. A, Phase-microscopy of living BT-20N cells. Bar, 10 μ m. B and C, Cells were grown on glass coverslips and processed for coimmunofluorescence localization with antibodies against E-cadherin (Jelly; B) and N-cadherin (C). D, BT-20 and BT-20N cells were extracted with NP-40 and 20 μ g protein from each extract was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E-cadherin (HECD1), N-cadherin, or P-cadherin. E, Extracts of BT-20N cells were immunoprecipitated with antibodies against N-cadherin or E-cadherin (HECD1). The immunoprecipitation reactions, as well as cell extracts, were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for N-cadherin and β -catenin (lanes 1 and 2) or E-cadherin (HECD1) and β -catenin (lanes 3 and 4).

with the parental cells. Although these cells expressed very high levels of E-cadherin, they did not display a typical epithelial morphology, and closely resembled the parent cell line (compare Figs. 6 A with 2 F). Both E- and N-cadherin were localized to regions of cell-cell contact (Fig. 6, B and C). When the MDA-MB-435E cells were tested for motility and invasion, they were not significantly different from the parental MDA-MB-435 cells (Fig. 5), even though β -catenin was associated with the transfected E-cadherin, as well as the endogenous N-cadherin (Fig. 6 E).

BT-20N Cells Effectively Segregate from HT1080 Fibroblasts

Hazan et al. (1997) suggested that N-cadherin-expressing breast cancer cells invade the stroma because they associate with the N-cadherin-expressing stromal cells. In our studies, we employed an *in vitro* invasion assay in which the cells invade an extracellular matrix that does not include any stromal cells. Thus, we can make the important statement that, in our studies, N-cadherin actively promotes invasion and motility. In Hazan et al. (1997), the investigators showed that N-cadherin-expressing breast cancer cells coaggregated with N-cadherin-expressing fibroblast-like cells. Since it has been suggested that it is the entire complement of cadherins expressed by a cell that determines its ability to associate with other cells, and that even cells expressing different levels of the same cadherin

can sort from one another (Steinberg and Takeichi, 1994), we sought to determine if the BT-20N cells that express N-, E-, and P-cadherin would segregate from an N-cadherin-expressing fibroblast cell line, HT1080. Equal numbers of BT-20 cells and HT1080 cells, or BT-20N cells and HT1080 cells, were mixed together and allowed to settle on glass coverslips. They were then prepared for immunofluorescence analysis using antibodies against E- or N-cadherin. In the immunofluorescence analysis of the BT-20/HT1080 cocultures, E-cadherin stained only the BT-20 cells and N-cadherin stained only the HT1080 cells. Fig. 7, A and B, show that these two cell lines effectively segregated from one another as expected. In the immunofluorescence analysis of the BT-20N/HT1080 cocultures, antibodies against E-cadherin stained only the BT-20N cells, whereas antibodies against N-cadherin stained both the BT-20N cells and the HT1080 cells. Fig. 7, C and D, show that the BT-20N cells and the HT1080 cells effectively segregated from one another, even though both cell lines express N-cadherin. Thus, epithelial cells that express N-cadherin along with other cadherins have not necessarily gained the ability to intermix with stromal cells.

Cadherin-11 Promotes Motility in Breast Epithelial Cells

In the course of our studies on breast tumor cell lines, we characterized one atypical line (MDA-MB-231) that did

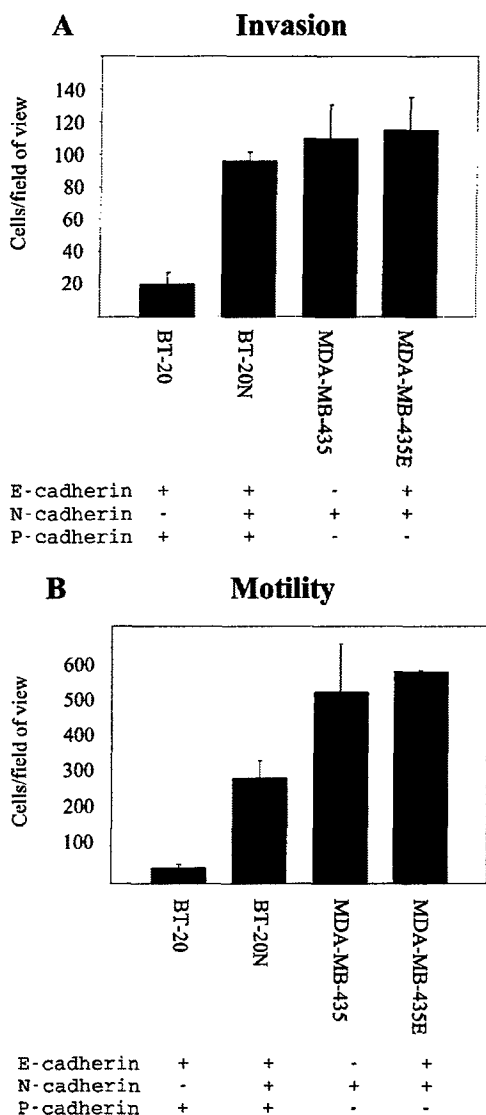


Figure 5. Exogenous expression of N-cadherin by BT-20 cells (BT-20N) increases their invasiveness, whereas exogenous expression of E-cadherin by MDA-MB-435 cells (MDA-MB-435E) does not effect their behavior. Cells were plated on Matrigel-coated or noncoated membranes for invasion assays or motility assays, respectively. The cells were incubated for 24 h, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transverse the membrane was determined by averaging ten random fields of view at 100 \times . The data are expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate SD of the average.

not express E-, P-, or N-cadherin, but nonetheless was invasive (Table I). Since MDA-MB-231 cells expressed significant levels of β -catenin, a protein that is not stable in cadherin-negative cells, we suspected that this cell line expressed another member of the cadherin family of pro-

teins, possibly one that is closely related to N-cadherin. We therefore analyzed RNA from this line with degenerate PCR primers designed to amplify all cadherins and found that it expressed cadherin-11 mRNA. Expression of cadherin-11 protein was confirmed by immunoblotting data with a cadherin-11-specific mAb, in agreement with recent data (Pishvaian et al., 1999). Like N-cadherin, cadherin-11 is expressed by some mesenchymal cells (Simoneau et al., 1995). Interestingly, cadherin-11 is expressed in some epithelial cells of the human placenta, and it has been suggested that cadherin-11 plays a role in mediating trophoblast-endometrium interactions as the cytotrophoblasts invade the uterine wall (MacCalman et al., 1996). Thus, one idea is that cadherin-11 could act in a manner similar to N-cadherin in promoting cell motility and invasion in breast cancer cells. To test this idea, we transfected cadherin-11 into BT-20 cells (BT-20Cad-11 cells). Like the BT-20N cells, BT-20Cad-11 cells retained the morphology of their parent line, even though they expressed high levels of cadherin-11 at cell-cell borders (Fig. 8, A-C). As predicted, cadherin-11-expressing BT-20 cells were more invasive and motile than the parental BT-20 cells (Fig. 8, D and E). Interestingly, the cadherin-11-expressing cells were not as invasive or motile as the N-cadherin-expressing cells. For example, the MDA-MB-231 cells were not as motile as the MDA-MB-435 cells (Figs. 5 and 8). More significantly, the BT-20 cells transfected with cadherin-11 did not become as motile as they did when transfected with N-cadherin. This may be due to differences between the two cadherins, or differences in expression levels of the transfected cadherins. It is reasonable to speculate that the level of expression of the inappropriate cadherin is relevant since the cell line SUM1315 expresses a small amount of cadherin-11, yet is not invasive.

N-Cadherin May Promote Cell Motility through a Fibroblast Growth Factor Receptor Signal Transduction Pathway

The laboratories of Frank Walsh and Patrick Doherty have shown that N-cadherin promotes neurite outgrowth from cerebellar neurons (Williams et al., 1994a). In addition, they showed that N-cadherin-mediated neurite extension was dependent on FGF receptor signaling, but was independent of ligand (Williams et al., 1994b). Walsh and Doherty thus proposed a model whereby the FGF receptor was induced to dimerize in the absence of FGF via interaction with N-cadherin (Doherty and Walsh, 1996). Dimerization of the FGF receptor results in receptor cross phosphorylation that initiates a number of signal transduction pathways. The pathway relevant to N-cadherin-dependent neurite outgrowth involves the generation of arachidonic acid from diacylglycerol, by the action of diacylglycerol lipase. The Walsh and Doherty laboratories showed that the diacylglycerol lipase inhibitor, RHC 80267, prevented neurite extension on N-cadherin-transfected 3T3 cells, thus implicating this type of FGF receptor signaling in N-cadherin-dependent neurite extension (Meiri et al., 1998). We hypothesized that the N-cadherin-mediated cell motility we observed in epithelial cells may also be acting through FGF receptor signaling. To test this hypothesis, we treated MDA-MB-435 cells, BT-20 cells,

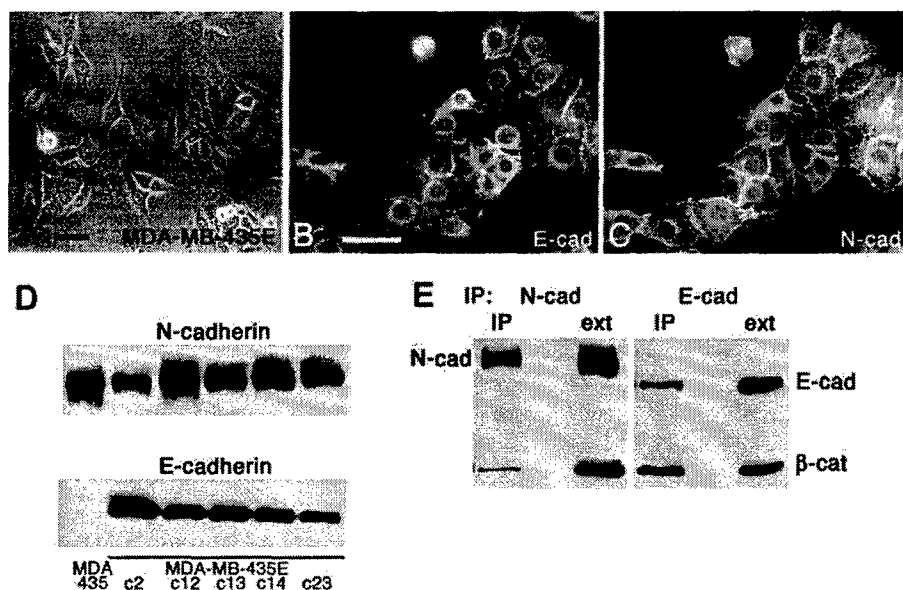


Figure 6. Expression of E-cadherin by MDA-MB-435 cells. MDA-MB-435 cells were transfected with E-cadherin (MDA-MB-435E) and expression was induced with dexamethasone. A, Phase-contrast microscopy of MDA-MB-435E cells. Bar, 10 μ m. B and C, Cells were grown on glass coverslips and processed for coimmunofluorescence localization with antibodies against E- (Jelly; B) and N-cadherin (C). D, MDA-MB-435 and several clones of MDA-MB-435E cells were extracted with NP-40 and 20 μ g protein from each extract was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for E- (HECD1) and N-cadherin. Clone 2 (c12) expressed the highest level of E-cadherin

and was chosen for subsequent studies. E, Extracts of MDA-MB-435 and MDA-MB-435E cells were immunoprecipitated with antibodies against N- or E-cadherin (HECD1). The immunoprecipitation reactions, along with cell extracts, were resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for N-cadherin and β -catenin (lanes 1 and 2), or E-cadherin (HECD1) and β -catenin (lanes 3 and 4).

and BT-20N cells with varying levels of RHC80267 to determine if it would influence the motility of these cells in the transwell assay. RHC80267 inhibited cell motility in both N-cadherin-expressing cell lines in a dose-dependent manner (Fig. 9 A). Importantly, this inhibitor had no effect on the motility of the N-cadherin-negative BT-20 cells. Although these data are consistent with the hypothesis that N-cadherin dependent cell motility is mediated

through FGF receptor signaling in a manner similar to N-cadherin-dependent neurite outgrowth, additional experiments must be done to further support this notion. Thus, we are continuing to investigate the mechanism whereby N-cadherin mediates motility in epithelial cells. To determine if cadherin-11 and N-cadherin promote cell motility through a similar pathway, we treated MDA-MB-231 and BT-20cad11 cells with RHC80267, and compared

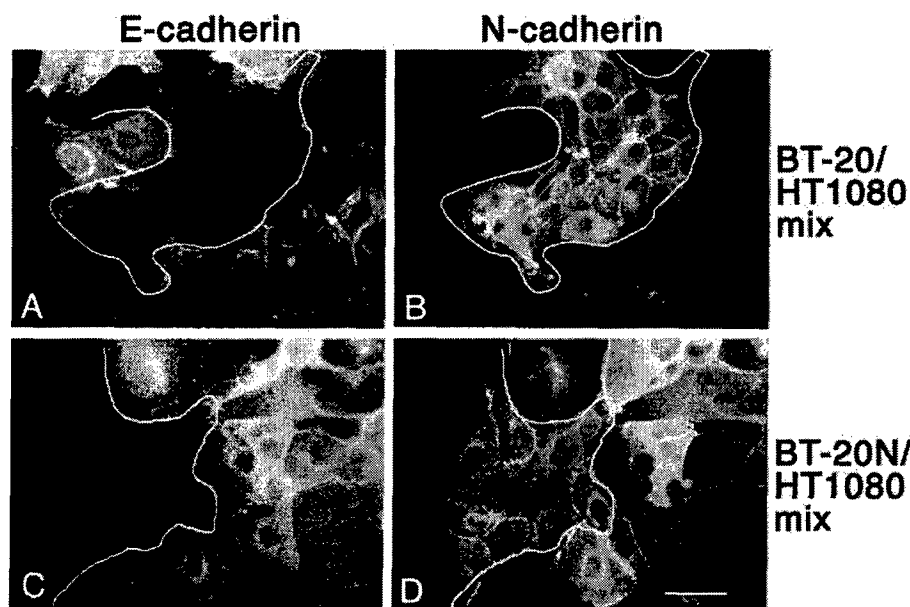


Figure 7. BT-20N cells do not mix with HT1080 cells. 5×10^4 BT-20 or BT-20N cells were mixed with an equal number of HT1080 cells, allowed to settle on coverslips, and processed for immunofluorescence with an mAb against N- (13A9) or E-cadherin (Jelly). A and B are a mix of BT-20 and HT1080 cells stained for E- and N-cadherin, respectively. The encircled cells are a group of E-cadherin-negative, N-cadherin-positive HT1080 cells. C and D are a mix of BT-20N and HT1080 cells stained for E- and N-cadherin, respectively. The encircled cells are a group of E-cadherin-negative, N-cadherin-positive HT1080 cells.

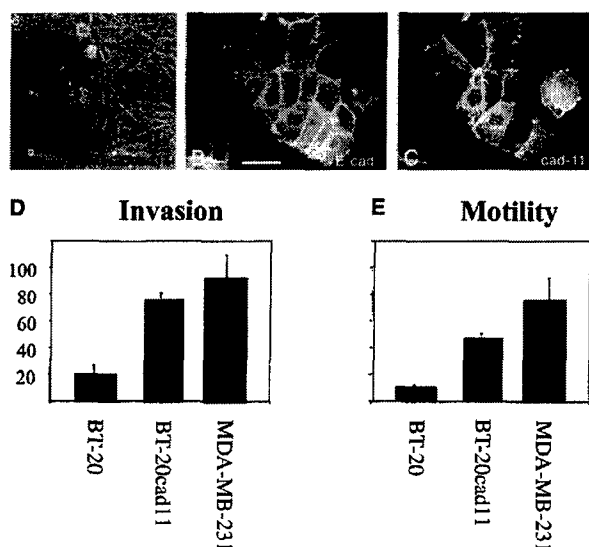


Figure 8. Exogenous expression of cadherin-11 by BT-20 cells (BT-20cad11) increases their invasiveness. BT-20 cells were transfected with cadherin-11 (BT-20cad11) and expression induced with dexamethasone. A, Phase-microscopy of living BT-20cad11 cells. Bars, 10 μ m. B and C, Cells were grown on glass coverslips and processed for coimmunofluorescence localization with antibodies against E-cadherin (Jelly; B) and cadherin-11 (C). D and E, Cells were plated on Matrigel-coated or noncoated membranes for invasion assays or motility assays, respectively. The cells were incubated for 24 h, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transverse the membrane was determined by averaging ten random fields of view at 100 \times . The data are expressed as the number of cells/field of view and is the average of three independent experiments. Error bars indicate SD of the average.

motility rates between treated and nontreated cells (Fig. 9 B). The diacylglycerol lipase inhibitor decreased the motility of cadherin-11-expressing cells in a dose-dependent manner. Cadherin-11-expressing cells are less motile than MDA-MB-435, and the inhibitor is less effective in decreasing the motility of the cadherin-11 expressing cells, suggesting there may be some differences in the respective signal transduction pathways, possibly in growth factor receptor levels or isoforms.

Discussion

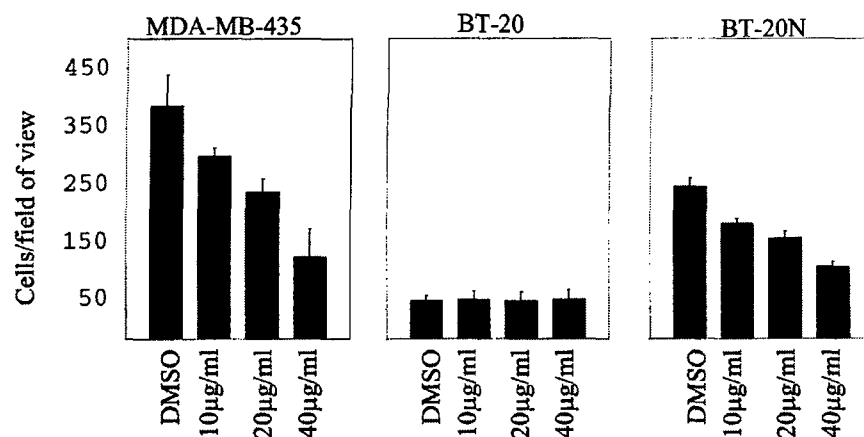
Previously, our laboratory showed that expression of different cadherin family members by squamous epithelial cells markedly affected morphology (Islam et al., 1996), i.e., when oral squamous epithelial cells expressed N-cadherin, they converted to a fibroblastic phenotype concurrent with decreased cell-cell adhesion. Thus, when we turned our attention to breast cancer cells for the present study, we were interested not only in the expression of various cadherins by these cells, but also in whether these cadherins influenced the morphology of the cells. We were not surprised to find that breast cancer cells endogenously

expressing N-cadherin displayed a fibroblastic phenotype with tenuous cell-cell contacts, whereas breast cancer cells endogenously expressing E-cadherin displayed a typical epithelial morphology. We were, however, surprised to find that transfection of N-cadherin into the E-cadherin-expressing BT-20 breast cancer cell line had no effect on morphology, even though it had a dramatic effect on cell behavior. Equally surprising was the fact that forced expression of E-cadherin had no effect on the morphology of the fibroblastic N-cadherin-expressing MDA-MB-435 cells. Thus, the breast cancer cell lines examined in this study behaved very differently from the oral squamous epithelial lines that we characterized previously. Interestingly, the oral squamous epithelial cells downregulated E-cadherin when they were forced to express N-cadherin, suggesting an inverse relationship between these cadherins. In contrast, the breast cancer cells continued to express their endogenous cadherin(s) when transfected with a different cadherin. The continued expression of endogenous cadherin may account for the lack of morphological change in the transfectants. Thus, the breast cancer cells differ from the oral squamous epithelial cells in two very important ways: first, the oral squamous epithelial cells appear to coregulate cadherins in an inverse manner, whereas these cadherins are independently regulated in breast cancer cells; and second, expression of E-cadherin by the oral squamous epithelial cells is sufficient for epithelial morphology, whereas epithelial morphology in the breast cancer cells appears to depend on other factors, in addition to E-cadherin.

In the present study, we have demonstrated that N-cadherin (or cadherin-11) expression in human breast carcinoma cells promotes an invasive phenotype. By transfecting the BT-20 cells with these nonepithelial cadherins, we have provided evidence for a direct role of these cadherins in cell motility and invasion. Previous studies have correlated the expression of N-cadherin or cadherin-11 with invasion in breast cancer cells. However, in this study, we took the important next step and used transfection studies to show that a cell line that has a low invasion rate could be converted to a highly invasive cell by expression of N-cadherin or cadherin-11. The BT-20 breast cancer cell line provided an important tool for these studies since they did not downregulate E-cadherin when forced to express N-cadherin. Thus, we can conclude that, even in cells expressing high levels of E-cadherin, N-cadherin (or cadherin-11) can promote motility, suggesting that, in this regard, both N-cadherin and cadherin-11 are dominant over E-cadherin. A study by Sommers et al. (1994) supports this idea. These authors showed that transfection of E-cadherin into the E-cadherin-negative breast cancer cell lines, BT549 and HS578, did not decrease the invasive capacity of these cells. These authors suggested that the transfected E-cadherin was not functional; however, these authors were unaware of the fact that the BT549 and HS578 cell lines express N-cadherin.

A previous study using MDA-MB-435 cells showed that transfection of E-cadherin into these cells reduced their capacity to form tumors when injected into the foot pads of nude mice (Meiners et al., 1998). In contrast to our study, these authors showed that E-cadherin-transfected clones of MDA-MB-435 cells underwent a morphological

A



B

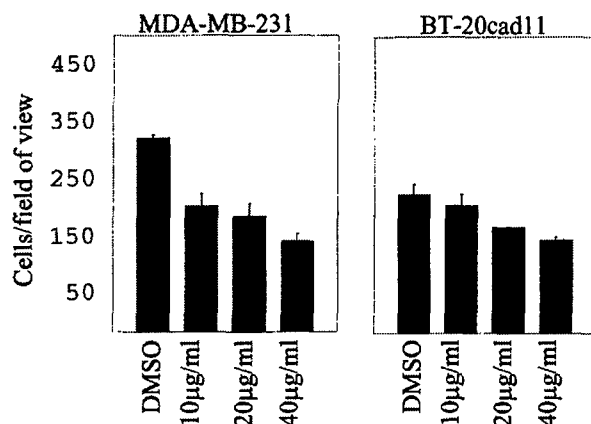


Figure 9. The diacylglycerol lipase inhibitor RHC80267 decreases motility of N-cadherin- and cadherin-11-expressing cells. Cells were plated on noncoated membranes for motility assays. The cells were incubated for 24 h in the presence of RHC80267 at varying concentrations, and those that did not migrate through the pores in the membrane were removed by scraping the membrane with a cotton swab. The remaining cells were stained, and the number transverse the membrane was determined by averaging ten random fields of view at 100 \times . The data are expressed as the number of cells/field of view and is the average of three (A) or two (B) independent experiments. Error bars indicate SD of the average.

change upon E-cadherin expression. In addition, they showed that E-cadherin-transfected clones were less tumorigenic in their assay than the parental cells. One difference in the study of Meiners et al. (1998) and ours is that they did not assay for N-cadherin expression in their E-cadherin-positive clones of MDA-MB-435 transfectants. Our study clearly demonstrates that N-cadherin influences the behavior of the cells, and that cells retaining N-cadherin do not undergo a morphological or behavioral change upon expression of E-cadherin. Thus, one possible explanation for the difference between these two studies is that the cells in the Meiners' study did not express N-cadherin. The point of our study was to determine if N-cadherin was capable of influencing the behavior of epithelial cells, even if they expressed E-cadherin, thus, we were particularly careful to select cell lines that retained N-cadherin expression after transfection with E-cadherin (Fig. 6).

One puzzling aspect of cell lines derived from metastatic

tumors is that they often express E-cadherin and appear to be relatively normal epithelial cells. A possibility suggested by the present study is that such cells may have up-regulated the expression of N-cadherin during the process of metastasis. Our results suggest that expression of N-cadherin would confer on these cells the capacity to invade, even though they continued to express E-cadherin. In this regard, expression of an inappropriate cadherin like N-cadherin (or other related cadherins) may be a better gauge of the clinical state of a tumor than is decreased expression of E-cadherin.

Some of the E-cadherin-negative breast cancer cells expressed endogenous P-cadherin. These cells had a fibroblastic morphology similar to that of the N-cadherin-expressing cells; however, they were not highly invasive, suggesting that P-cadherin confers upon breast cancer cells characteristics different from those conferred by either E- or N-cadherin. P-cadherin is expressed in the myo-

epithelial cells surrounding the luminal epithelial cells of the mammary gland. Radice et al. (1997) recently showed that P-cadherin deficient mice develop age-dependent hyperplasia and dysplasia of the mammary epithelium, and suggested that P-cadherin may play a role in maintaining the normal phenotype of breast epithelial cells. One possibility is that the P-cadherin-expressing tumor cells were derived from the myoepithelium, rather than from the true epithelium.

E-cadherin has been termed an invasion suppressor because transfection of this protein into some E-cadherin-negative invasive carcinoma cells resulted in decreased invasive capacity. Our prediction is that at least some of these cell lines expressed a cadherin, like N-cadherin or cadherin-11, and overexpression of E-cadherin resulted in downregulation of the endogenous cadherin, as we saw with the oral squamous epithelial cells. Thus, we hypothesize that the invasion suppressor role of E-cadherin arises in part from its ability to decrease the level of N-cadherin in certain, but not all, tumors. In the present study, cell lines that did not express any classical cadherins, as evidenced by lack of β -catenin protein, as well as lack of detectable cadherin, had low invasion rates. Our hypothesis, that loss of E-cadherin alone does not necessarily increase invasive capacity in breast carcinoma cells, is supported by the observation that function-blocking antibodies against E-cadherin did not confer a highly motile, invasive phenotype on MCF-7 cells, a breast cancer cell line that is E-cadherin-positive and N-cadherin-negative (Sommers et al., 1991). The current study suggests that, in some carcinoma cells, expression of N-cadherin, or a similar cadherin such as cadherin-11, may actually be necessary for increased motility and invasion. A recent clinical study suggested that inactivation of E-cadherin is an early event in the progression of lobular breast carcinomas (Vos et al., 1997). We might suggest that a subsequent event would be activation of the expression of an inappropriate cadherin, such as N-cadherin or cadherin-11.

Understanding the mechanism by which N-cadherin promotes motility in epithelial cells is important if we are to develop treatments that will decrease the invasiveness of tumor cells. A number of studies have shown that epithelial cells can be induced to scatter in response to growth factors, such as hepatocyte growth factor and members of the FGF, EGF, and TGF families (Blay and Brown, 1985; Vallés et al., 1990; Behrens et al., 1991; Geimer and Bade, 1991; Gherardi and Stoker, 1991; Rosen et al., 1991; Miettinen et al., 1994; Savagner et al., 1994, 1997). Walsh, Doherty, and coworkers have established, through extensive studies on FGF receptor and cell adhesion molecules, that N-cadherin and the FGF receptor cooperate to induce neurite outgrowth in cerebellar neurons (reviewed in Doherty and Walsh, 1996; Walsh and Doherty, 1997). These authors have proposed a scheme for activation of the kinase activity of the FGF receptor through cis interactions with N-cadherin, via an HAV domain in the FGF receptor and an HAV interaction domain in the fourth extracellular domain of N-cadherin (Doherty and Walsh, 1996). In addition, it has been proposed that the cadherins form lateral dimers in the plane of the membrane (Shapiro et al., 1995; Takeda et al., 1999), which could result in dimerization of the FGF receptor, and subsequent activa-

tion of the signal transduction pathway. We based the studies presented herein on the model presented by Walsh and Doherty, and proposed that interaction of N-cadherin with the FGF receptor in N-cadherin-expressing epithelial cells may result in increased motility, similar to that seen by treating epithelial cells with growth factors. To test this hypothesis, we interfered with the N-cadherin-dependent FGF receptor signal transduction pathway proposed by Walsh and Doherty by inhibiting a downstream enzyme, diacylglycerol lipase. We showed that inhibiting diacylglycerol lipase decreased motility of N-cadherin-expressing cells in a dose-dependent manner while having no effect on the motility of N-cadherin-negative cells. Thus, our data strongly support the notion that N-cadherin promotes motility in breast cancer cells by activating growth factor receptor signal transduction pathways. Continued efforts in our laboratory are aimed at further defining the signal transduction pathway(s) that mediate cadherin-dependent motility in epithelial cells.

At first glance, it might seem unlikely that expression of an additional cell adhesion molecule would confer a motile and invasive phenotype upon an epithelial cell. However, motile cells, such as fibroblasts and myoblasts, express N-cadherin (Knudsen et al., 1995; Huttenlocher et al., 1998) and a switch from E- to N-cadherin occurs in the chick embryo when epiblast cells ingress through the primitive streak to form the mesoderm (Edelman et al., 1983; Hatta and Takeichi, 1986). Another interesting cadherin switch occurs during establishment of the human placenta, where fetal cytotrophoblast cells invade the vasculature of the uterus. During this invasive process, the cytotrophoblast cells downregulate the expression of E-cadherin and upregulate vascular/endothelial (VE) cadherin (Zhou et al., 1997). Thus, it is feasible to suggest that increased expression of a nonepithelial cell cadherin, such as N-cadherin, could increase the invasive potential of tumor cells. Ongoing studies in our laboratory are designed to determine how N-cadherin differs from E-cadherin in its ability to induce cell motility. We hypothesize that E-cadherin does not have the ability to interact with the relevant growth factor receptors, and we are preparing chimeric molecules between E- and N-cadherin to test this hypothesis.

An important message from the present studies is that cadherins may not function identically in different cell types. The fact that cadherins may act differently in different cell types is particularly evident when comparing the current study with earlier studies showing that mouse L cells or S180 fibroblasts attained an epithelial morphology when transfected with either E- or N-cadherin (Nagafuchi et al., 1987; Hatta et al., 1988; Matsuzaki et al., 1990). It will be important in future studies to consider the cellular makeup, as well as the complement of cadherin family members, when interpreting data on cellular morphology and behavior.

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References

- Aberle, H., S. Butz, J. Stappert, H. Weissig, R. Kemler, and H. Hoschuetzky. 1994. Assembly of the cadherin-catenin complex in vitro with recombinant proteins. *J. Cell Sci.* 107:3655-3663.
- Aono, S., S. Nakagawa, A.B. Reynolds, and M. Takeichi. 1999. p120(ctn) acts as an inhibitory regulator of cadherin function in colon carcinoma cells. *J. Cell Biol.* 145:551-562.
- Behrens, J., M. Mareel, F.M. van Roy, and W. Birchmeier. 1989. Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. *J. Cell Biol.* 108:2435-2447.
- Behrens, J., K.M. Weidner, U.H. Frixen, J.H. Schipper, M. Sachs, N. Arakaki, Y. Daikuhara, and W. Birchmeier. 1991. The role of E-cadherin and scatter factor in tumor invasion and cell motility. *Exper. Suppl.* 59:109-126.
- Berx, G., A.M. Cleiton-Jansen, F. Nollet, W.J.F. de Leeuw, M.J. van de Vijver, M.J. Cornelisse, and F. van Roy. 1995. E-cadherin is a tumor/invasion suppressor gene mutated in human lobular breast cancers. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:6107-6115.
- Berx, G., A.M. Cleiton-Jansen, K. Strumane, W.J. de Leeuw, F. Nollet, F. van Roy, and C. Cornelisse. 1996. E-cadherin is inactivated in a majority of invasive human lobular breast cancers by truncation mutations throughout its extracellular domain. *Oncogene* 13:1919-1925.
- Blay, J., and K.D. Brown. 1985. Epidermal growth factor promotes the chemotactic migration of cultured rat intestinal epithelial cells. *J. Cell. Physiol.* 124:107-112.
- Bringuier, P.P., R. Umbas, H.E. Schaafsma, H.F. Karthaus, F.M. Debruyne, and J.A. Schalken. 1993. Decreased E-cadherin immunoreactivity correlates with poor survival in patients with bladder tumors. *Cancer Res.* 53:3241-3245.
- Chen, H., N. Paradies, M. Fedor-Chaiken, and R. Brackenbury. 1997. E-cadherin mediates adhesion and suppresses cell motility via distinct mechanisms. *J. Cell Sci.* 110:345-356.
- Chen, W.C., and B. Öbrink. 1991. Cell-cell contacts mediated by E-cadherin (uvomorulin) restrict invasive behavior of L-cells. *J. Cell Biol.* 114:319-327.
- Daniel, J.M., and A.B. Reynolds. 1995. The tyrosine kinase substrate p120^{cas} binds directly to E-cadherin but not to the adenomatous polyposis coli protein or α -catenin. *Mol. Cell Biol.* 15:4819-4824.
- Doherty, P., and F.S. Walsh. 1996. CAM-FGF receptor interactions: a model for axonal growth. *Mol. Cell Neurosci.* 8:99-111.
- Dorudi, S., J.P. Sheffield, R. Poulosom, J.M. Northover, and I.R. Hart. 1993. E-cadherin expression in colorectal cancer. An immunocytochemical and in situ hybridization study. *Am. J. Pathol.* 142:981-986.
- Edelman, G.M., W.J. Gallin, A. Delouree, B.A. Cunningham, and J.P. Thiery. 1983. Early epochal maps of two different cell adhesion molecules. *Proc. Natl. Acad. Sci. USA.* 80:4384-4388.
- Frixen, U.H., J. Behrens, M. Sachs, G. Eberle, B. Voss, A. Warda, D. Lochner, and W. Birchmeier. 1991. E-cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J. Cell Biol.* 113:173-185.
- Camello, C., J. Palacios, A. Suarez, A. Pizarro, P. Navarro, M. Quintanilla, and A. Cano. 1993. Correlation of E-cadherin expression with differentiation grade and histological grade in breast carcinoma. *Am. J. Pathol.* 142:987-993.
- Geimer, P., and E.G. Bade. 1991. The epidermal growth factor-induced migration of rat liver epithelial cells is associated with a transient inhibition of DNA synthesis. *J. Cell Sci.* 100:349-355.
- Gherardi, E., and M. Stoker. 1991. Hepatocyte growth factor-scatter factor: mitogen, motogen, and met. *Cancer Cells* 3:227-232.
- Guriec, N., L. Marcellin, B. Gairard, H. Calderoll, A. Wilk, R. Renaud, J.P. Bergerat, and F. Oberling. 1996. E-cadherin mRNA expression in breast carcinomas correlates with overall and disease-free survival. *Invasion Metastasis* 16:19-26.
- Hamaguchi, M., N. Matsuyoshi, Y. Ohnishi, B. Gotoh, M. Takeichi, and Y. Nagai. 1993. p60v-src causes tyrosine phosphorylation and inactivation of the N-cadherin-catenin cell adhesion system. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:307-314.
- Hatta, K., and M. Takeichi. 1986. Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. *Nature* 320:447-449.
- Hatta, K., A. Nose, A. Nagafuchi, and M. Takeichi. 1988. Cloning and expression of cDNA encoding a neural calcium-dependent cell adhesion molecule: its identity in the cadherin gene family. *J. Cell Biol.* 106:873-881.
- Hazan, R.B., L. Kang, B.P. Whooley, and P.I. Borgen. 1997. N-cadherin promotes adhesion between invasive breast cancer cells and the stroma. *Cell Adhes. Commun.* 4:399-411.
- Huttenlocher, A., M. Lakonishok, M. Kinder, S. Wu, T. Truong, K.A. Knudsen, and A.F. Horwitz. 1998. Integrin and cadherin synergy regulates contact inhibition of migration and motile activity. *J. Cell Biol.* 141:515-526.
- Islam, S., T.E. Carey, G.T. Wolf, M.J. Wheelock, and K.R. Johnson. 1996. Expression of N-cadherin by human squamous carcinoma cells induces a scattered fibroblastic phenotype with disrupted cell-cell adhesion. *J. Cell Biol.* 135:1643-1654.
- Johnson, K.R., J.E. Lewis, D. Li, J. Wahl, A.P. Soler, K.A. Knudsen, and M.J. Wheelock. 1993. P- and E-cadherin are in separate complexes in cells expressing both cadherins. *Exp. Cell Res.* 207:252-260.
- Knudsen, K.A., A.P. Soler, K.R. Johnson, and M.J. Wheelock. 1995. Interaction of α -actinin with the cadherin/catenin cell-cell adhesion complex via α -catenin. *J. Cell Biol.* 130:67-77.
- Lewis, J.E., P.J. Jensen, K.R. Johnson, and M.J. Wheelock. 1994. E-cadherin mediates adherens junction organization through protein kinase C. *J. Cell Sci.* 107:3615-3621.
- Lewis, J.E., J.K. Wahl III, K.M. Sass, P.J. Jensen, K.R. Johnson, and M.J. Wheelock. 1997. Cross-talk between adherens junctions and desmosomes depends on plakoglobin. *J. Cell Biol.* 136:919-934.
- Luo, J., D.M. Lubaroff, and M.J.C. Hendrix. 1999. Suppression of prostate cancer invasive potential and matrix metalloproteinase activity by E-cadherin transfection. *Cancer Res.* 59:3552-3556.
- MacCalman, C.D., E.E. Furth, A. Omigbodun, M. Bronner, C. Coutifaris, and J.F. Strauss III. 1996. Regulated expression of cadherin-11 in human epithelial cells: a role for cadherin-11 in trophoblast-endometrium interactions? *Dev. Dyn.* 206:201-211.
- Matsuyoshi, N., M. Hamaguchi, S. Tanaguchi, A. Nagafuchi, S. Tsukita, and M. Takeichi. 1992. Cadherin-mediated cell-cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts. *J. Cell Biol.* 118:703-714.
- Matsuzaki, F., R.M. Mege, S.H. Jaffe, D.R. Friedlander, W.J. Gallin, J.I. Goldberg, B.A. Cunningham, and G.M. Edelman. 1990. cDNAs of cell adhesion molecules of different specificity induce changes in cell shape and border formation in cultured S180 cells. *J. Cell Biol.* 110:1239-1252.
- Mayer, B., J.P. Johnson, F. Leitl, K.W. Jauch, M.M. Heiss, F.W. Schildberg, W. Birchmeier, and I. Funke. 1993. E-cadherin expression in primary and metastatic gastric cancer: downregulation correlates with cellular dedifferentiation and glandular disintegration. *Cancer Res.* 53:1690-1695.
- Meiners, S., V. Brinkmann, H. Naundorf, and W. Birchmeier. 1998. Role of morphogenetic factors in metastasis of mammary carcinoma cells. *Oncogene* 16:9-20.
- Meiri, K.F., J.L. Saffell, F.S. Walsh, and P. Doherty. 1998. Neurite outgrowth stimulated by neural cell adhesion molecules requires growth-associated protein-43 (GAP-43) function and is associated with GAP-43 phosphorylation in growth cones. *J. Neurosci.* 15:10429-10437.
- Miettinen, P.J., R. Ebner, A.R. Lopez, and R. Derynck. 1994. TGF- β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *J. Cell Biol.* 127:2021-2036.
- Moll, R., M. Mitze, U.H. Frixen, and W. Birchmeier. 1993. Differential loss of E-cadherin expression in infiltrating ductal and lobular breast carcinomas. *Am. J. Pathol.* 143:1731-1742.
- Nagafuchi, A., Y. Shirayoshi, K. Okazaki, K. Yasuda, and M. Takeichi. 1987. Transformation of cell adhesion properties by exogenously introduced E-cadherin cDNA. *Nature* 329:341-343.
- Nagafuchi, A., S. Ishihara, and S. Tsukita. 1994. The roles of catenins in the cadherin-mediated cell adhesion: functional analysis of E-cadherin- α -catenin fusion molecules. *J. Cell Biol.* 127:235-245.
- Nieset, J.E., A.R. Redfield, F. Jin, K.A. Knudsen, K.R. Johnson, and M.J. Wheelock. 1997. Characterization of the interactions of α -catenin with α -actinin and β -catenin/plakoglobin. *J. Cell Sci.* 110:1013-1022.
- Ohkubo, T., and M. Ozawa. 1999. p120(ctn) binds to the membrane-proximal region of the E-cadherin cytoplasmic domain and is involved in modulation of adhesion activity. *J. Biol. Chem.* 274:21409-21415.
- Oka, H., H. Shiozaki, K. Kobayashi, M. Inoue, H. Tahara, T. Kobayashi, Y. Takatsuka, N. Matsuyoshi, S. Hirano, M. Takeichi, and T. Mori. 1993. Expression of E-cadherin cell adhesion molecules in human breast cancer tissues and its relationship to metastasis. *Cancer Res.* 53:1696-1701.
- Okazaki, M., S. Takeshita, S. Kawai, R. Kikuno, A. Tsujimura, A. Kudo, and E. Amann. 1994. Molecular cloning and characterization of OB-cadherin, a new member of cadherin family expressed in osteoblasts. *J. Biol. Chem.* 269:12092-12098.
- Pierceall, W.E., A.S. Woodard, J.S. Morrow, D. Rimm, and E.R. Fearon. 1995. Frequent alterations in E-cadherin and α - and β -catenin expression in human breast cancer cell lines. *Oncogene* 11:1319-1326.
- Pishvalan, M.J., C.M. Feltes, P. Thompson, M.J. Bussemakers, J.A. Schalken, and S.W. Byers. 1999. Cadherin-11 is expressed in invasive breast cancer cell lines. *Cancer Res.* 59:947-952.
- Radice, G.L., M.C. Ferreira-Cornwell, S.D. Robinson, H. Rayburn, L.A. Chodosh, M. Takeichi, R.O. Hynes. 1997. Precocious mammary gland development in P-cadherin-deficient mice. *J. Cell Biol.* 139:1025-1032.
- Rasbridge, S.A., C.E. Gillett, S.A. Sampson, F.S. Walsh, and R.R. Millis. 1993. Epithelial (E-) and placental (P-) cadherin cell adhesion molecule expression in breast carcinoma. *J. Pathol.* 169:245-250.
- Reynolds, A.B., J. Daniel, P.D. McCrea, M.J. Wheelock, J. Wu, and Z. Zhang. 1994. Identification of a new catenin: the tyrosine kinase substrate p120^{cas} associates with E-cadherin complexes. *Mol. Cell Biol.* 14:8333-8342.
- Rimm, D.L., E.R. Koslov, P. Kebriaei, C.D. Cianci, and J.S. Morrow. 1995. α (E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. *Proc. Natl. Acad. Sci.*

- Sci. USA.* 92:8813-8817.
- Rosen, E.M., J. Knesel, and I.D. Goldberg. 1991. Scatter factor and its relationship to hepatocyte growth factor. *Cell Growth Differ.* 2:603-607.
- Sacco, P.A., T.M. McGranahan, M.J. Wheelock, and K.R. Johnson. 1995. Identification of plakoglobin domains required for association with N-cadherin and α -catenin. *J. Biol. Chem.* 270:20201-20206.
- Savagner, P., A.M. Vallés, J. Jouanneau, K.M. Yamada, and J.P. Thiery. 1994. Alternative splicing in fibroblast growth factor receptor 2 is associated with induced epithelial-mesenchymal transition in rat bladder carcinoma cells. *Mol. Biol. Cell.* 5:851-862.
- Savagner, P., K.M. Yamada, and J.P. Thiery. 1997. The zinc-finger protein slug causes desmosome dissociation, an initial and necessary step for growth factor-induced epithelial-mesenchymal transition. *J. Cell Biol.* 137:1403-1419.
- Schipper, J.H., U.H. Frixen, J. Behrens, A. Unger, K. Jahnke, and W. Birchmeier. 1991. E-cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. *Cancer Res.* 51:6328-6337.
- Shapiro, L., A.M. Fannon, P.D. Kwong, A. Thompson, M.S. Lehman, G. Grubel, J.F. Legrand, J. Als-Nielsen, D.R. Colman, and W.A. Hendrickson. 1995. Structural basis of cell-cell adhesion by cadherins. *Nature.* 374:327-337.
- Shibamoto, S., M. Hayakawa, K. Takeuchi, T. Hori, K. Miyazawa, N. Kitamura, K.R. Johnson, M.J. Wheelock, N. Matsuyoshi, M. Takeichi, and F. Ito. 1995. Association of p120, a tyrosine kinase substrate, with E-cadherin/catenin complexes. *J. Cell Biol.* 128:949-957.
- Simonneau, L., M. Kitagawa, S. Suzuki, and J.P. Thiery. 1995. Cadherin 11 expression marks the mesenchymal phenotype: towards new functions for cadherins? *Cell Adhes. Commun.* 3:115-130.
- Sommers, C.L., E.W. Thompson, J.A. Torri, R. Kemler, E.P. Gelmann, and S.W. Byers. 1991. Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacities. *Cell Growth Differ.* 2:365-372.
- Sommers, C.L., E.P. Gelmann, R. Kemler, P. Cowin, and S.W. Byers. 1994. Alterations in β -catenin phosphorylation and plakoglobin expression in human breast cancer cells. *Cancer Res.* 54:3544-3552.
- Stappert, J., and R. Kemler. 1994. A short core region of E-cadherin is essential for catenin binding and is highly phosphorylated. *Cell Adhes. Commun.* 2:319-327.
- Steinberg, M.S., and M. Takeichi. 1994. Experimental specification of cell sorting, tissue spreading, and specific spatial patterning by quantitative differences in cadherin expression. *Proc. Natl. Acad. Sci. USA.* 91:206-209.
- Takeda, H., Y. Shimoyama, A. Nagafuchi, and S. Hirohashi. 1999. E-cadherin functions as a cis-dimer at the cell-cell adhesive interface in vivo. *Nat. Struct. Biol.* 6:310-312.
- Umbas, R., W.B. Isaacs, P.P. Bringuier, H.E. Schaafsma, H.F. Karthaus, G.O. Oosterhof, F.M. Debruyne, and J.A. Schalken. 1994. Decreased E-cadherin expression is associated with poor prognosis in patients with prostate cancer. *Cancer Res.* 54:3929-3933.
- Valles, A.M., B. Boyer, J. Badet, G.C. Tucker, D. Barritault, and J.P. Thiery. 1990. Acidic fibroblast growth factor is a modulator of epithelial plasticity in a rat bladder carcinoma cell line. *Proc. Natl. Acad. Sci. USA.* 87:1124-1128.
- Vos, C.B., A.M. Cleton-Jansen, G. Berx, W.J. de Leeuw, N.T. ter Haar, F. van Roy, C.J. Cornelisse, J.L. Peterse, and M.J. van de Vijver. 1997. E-cadherin inactivation in lobular carcinoma in situ of the breast: an early event in tumorigenesis. *Br. J. Cancer.* 76:1131-1133.
- Walsh, F.S., and P. Doherty. 1997. Neural cell adhesion molecules of the immunoglobulin super family: role in axonal growth and guidance. *Annu. Rev. Cell Biol.* 13:425-456.
- Watabe-Uchida, M., N. Uchida, Y. Imamura, A. Nagafuchi, K. Fujimoto, T. Uemura, S. Vermeulen, F. van Roy, E.D. Adamson, and M. Takeichi. 1998. α -Catenin-vinculin interaction functions to organize the apical junctional complex in epithelial cells. *J. Cell Biol.* 142:847-857.
- Wheelock, M.J., C.A. Buck, K.B. Bechtol, and C.H. Damsky. 1987. Soluble 80-kd fragment of cell-CAM 120/80 disrupts cell-cell adhesion. *J. Cell Biochem.* 34:187-202.
- Wheelock, M.J., K.A. Knudsen, and K.R. Johnson. 1996. Membrane-cytoskeleton interactions with cadherin cell adhesion proteins: roles of catenins as linker proteins. *Curr. Top. Membr.* 43:169-185.
- Williams, E.J., F.S. Walsh, and P. Doherty. 1994a. Tyrosine kinase inhibitors can differentially inhibit integrin-dependent and CAM-stimulated neurite outgrowth. *J. Cell Biol.* 124:1029-1037.
- Williams, E.J., J. Furness, F.S. Walsh, and P. Doherty. 1994b. Activation of the FGF receptor underlies neurite outgrowth stimulated by L1, N-CAM, and N-cadherin. *Neuron.* 13:583-594.
- Zhou, Y., S.J. Fisher, M. Janatpour, O. Genbacev, E. Dejana, M.J. Wheelock, and C.H. Damsky. 1997. Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *J. Clin. Invest.* 99:2139-2151.